

Metabolic chamber studies
on energy- and macronutrient metabolism:
Impact of meal skipping and energy flux

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*"I do not know what I may appear to the world;
but to myself I seem to have been only like a boy playing on the seashore,
and diverting myself in now
and then finding a smoother pebble or a prettier shell than ordinary,
whilst the great ocean of truth lay all undiscovered before me."*

- Isaac Newton -

Abstract

The classical concept of body weight regulation attributes the development of obesity to a chronically positive energy balance. There is, however, evidence indicating that beyond this basic concept, the effectiveness of body weight regulation is affected by the circadian regulation of metabolism and the level of energy flux (EF, level of energy balance). Meal skipping affects circadian regulation and might therefore also affect the regulation of body weight. In addition, an asymmetric regulation of body weight is hypothesized with improved effectiveness when EF is high (active lifestyle) and less effectiveness at a low EF (sedentary lifestyle). Metabolic chambers offer the opportunity to acquire short-term parameters of energy and macronutrient balance that precede long-term weight gain and therefore, can help to understand the impact of nutrition and physical activity interventions on body weight regulation.

This thesis presents the implementation of a metabolic chamber system (*Chapter II*) and investigates the acute impact of meal skipping (*Chapter III*) and energy flux (*Chapter IV*) on energy and macronutrient metabolism by performing two well-controlled, cross-over intervention studies using metabolic chambers.

The implementation of the metabolic chambers revealed, that thorough considerations must be made in terms of the metabolic chamber environment (room ventilation and position of analyzer unit), the additional devices (e.g. air conditioner) used as well as the study protocol, in order to obtain good data quality.

The study on meal skipping includes 17 healthy participants who underwent 3 isocaloric 24-h interventions (55%, 30%, and 15% carbohydrate, fat and protein, respectively): a breakfast skipping day (BSD) and a dinner skipping day (DSD) separated by a conventional 3-meal-structure day (control). Energy and macronutrient balance were measured and postprandial glucose and insulin concentrations, as well as 24-h glycemia and 24-h insulin secretion (C-peptide), were analyzed.

When compared with the 3-meal control, 24-h energy expenditure was higher on DSD (DSD: +69 kcal/d; $p < 0.05$), but not on BSD. Whereas, fat oxidation increased on the BSD only (+13 g/d; $p < 0.01$). Spontaneous physical activity, 24-h glycemia, and 24-h insulin secretion did not differ between intervention days. The postprandial homeostasis model

assessment index (+54%) and glucose concentrations after lunch (+46%) were, however, higher on the BSD than on the DSD (both $p < 0.05$).

When compared with 3 meals/d, dinner skipping increased energy expenditure. In contrast, higher postprandial insulin concentrations and increased fat oxidation with breakfast skipping show the development of metabolic inflexibility in response to prolonged fasting that may in the long-term lead to impaired glucose homeostasis.

The study on energy flux includes 16 healthy participants who underwent three 24-h interventions with different levels of EF: (i) low EF, physical activity level (PAL) = 1.3 – 1.4 (ii) medium EF, PAL = 1.5 – 1.6 and (iii) high EF, PAL = 1.7 – 1.8 each at energy balance (EB), caloric restriction (CR), and overfeeding (OF) (100%, 75% and 125% of individual energy requirement with 50% carbohydrate, 35% fat, 15% protein). Different levels of EF were accomplished by walking (4 km/h) on a treadmill (0, 165 and 330 min). Sleeping energy expenditure (SEE), 24-h macronutrient oxidation and relative macronutrient balance (oxidation relative to intake) were determined.

During EB and OF, 24-h fat oxidation increased with higher EF. This resulted in a higher relative fat balance at medium EF (EB: +17%, OF: +14%) and high EF (EB: +23%, OF: +17%) compared to low EF (all $p < 0.05$). SEE during EB and OF was higher at medium (EB: +5 kcal/3h and OF: +12 kcal/3h) and high (EB: +7 kcal/3h and OF: +18 kcal/3h) EF compared to low EF (all, $p < 0.05$). In contrast, during CR 24-h fat oxidation was only higher at high EF compared to low EF and neither relative fat balance nor SEE differed between the EF levels.

A higher EF might have beneficial effects on body weight regulation during short-term overfeeding and energy balance because it increased SEE and improved relative fat balance. However, during short-term caloric restriction, a higher EF had no impact on the regulation of energy or fat balance. Therefore, a high EF especially can attenuate the adverse effects of short-term overfeeding.

Altogether, this thesis emphasizes the importance of physical activity in daily life and suggests that the adverse metabolic outcome of breakfast skipping (caused by a positive energy balance after lunch with a preceding prolonged fasting period) might be attenuated by a high EF.

Zusammenfassung

Das klassische Konzept der Regulation des Körpergewichtes begründet die Entwicklung von Adipositas mit einer chronisch positiven Energiebilanz. Es gibt Hinweise, dass die Effektivität, das Körpergewicht zu regulieren, darüber hinaus durch die zirkadiane Regulation des Stoffwechsels und die Höhe des *Energy-Flux* (EF, Niveau der Energiebilanz) beeinflusst wird. Das Auslassen von Mahlzeiten kann die zirkadiane Regulation und somit möglicherweise das Körpergewicht beeinflussen. Zudem wird eine asymmetrische Regulation des Körpergewichtes angenommen mit einer höheren Effektivität bei einem hohen EF (aktiver Lebensstil) verglichen mit einem niedrigen EF (inaktiver Lebensstil). Die Raumkalorimetrie bietet die Möglichkeit kurzfristige Parameter der Energie- und Makronährstoffbilanz zu erfassen und kann somit helfen, den Einfluss von Ernährungsinterventionen und von körperlicher Aktivität auf die Körpergewichtsregulation zu verstehen.

Diese Arbeit hat zum Ziel, die Implementierung eines Raumkalorimeters zu demonstrieren (*Kapitel II*), sowie durch zwei gut kontrollierte *cross-over*-Interventionen zum einen die akuten Auswirkungen des Auslassens von Mahlzeiten (*Kapitel III*) und zum anderen die Auswirkungen des *Energy-Flux* (*Kapitel IV*) auf den Energie- und Makronährstoffmetabolismus, zu untersuchen.

Die Implementierung der Raumkalorimetrie konnte zeigen, dass es sorgfältiger Überlegungen bedarf, bezüglich der Umgebung der Raumkalorimeter (Raumlüftung, Positionierung des Gasanalysators), zusätzlich verwendeter Geräte (z.B. Klimaanlage) und des Studienprotokolls, um Daten einer guten Qualität generieren zu können.

Die Studie zum Auslassen von Mahlzeiten schließt 17 gesunde Probanden ein, die 3 isokalorische Protokolle durchliefen (jeweils 55%, 30%, und 15% Kohlenhydrate, Fett und Protein): ein Frühstückskipping Tag (BSD) und ein Abendessen-Skippping Tag (DSD) sowie einen Tag mit einer gewöhnlichen Drei-Mahlzeiten-Struktur (*control*). Die Energie- und Makronährstoffbilanz wurde gemessen und die postprandialen Glukose- und Insulinkonzentrationen sowie die 24-h Glykämie und 24-h Insulinsekretion (C-Peptid-Ausscheidung) wurden analysiert.

Verglichen mit der 3-Mahlzeiten-Kontrolle war der 24-h Energieverbrauch am DSD höher (DSD: +69 kcal/d; $p < 0.05$), aber nicht am BSD. Hingegen war die 24-h Fettoxidation nur am BSD erhöht (+13 g/d; $p < 0.01$). Spontane körperliche Aktivität, 24-h Glykämie und 24-h Insulinsekretion unterschieden sich nicht zwischen den

Interventionstagen. Der postprandiale *HOMA-Index* (+54%) und die Glukosekonzentration nach dem Mittagessen (+46%) waren jedoch am BSD höher als am DSD (beide $p < 0.05$).

Das Auslassen des Abendessens erhöhte den Energieverbrauch verglichen mit drei Mahlzeiten/Tag. Höhere postprandiale Insulinkonzentrationen in Verbindung mit einer erhöhten Fettoxidation durch das Auslassen des Frühstücks zeigen die Entwicklung einer metabolischen Inflexibilität als Reaktion auf eine verlängerte Nüchternphase, was langfristig zur Beeinträchtigung der Glukose-Homöostase führen könnte.

Die Studie zum *Energy-Flux* umfasst 16 gesunde Probanden, die drei 24-h Interventionen mit verschiedenen EF Niveaus durchliefen: (i) niedriger EF, *physical activity level* (PAL) = 1.3 – 1.4 (ii) mittlerer EF, PAL = 1.5 – 1.6 und (iii) hoher EF, PAL = 1.7 – 1.8, jeweils unter ausgeglichener Energiebilanz (EB), Kalorienrestriktion (CR) und Überernährung (OF) (100%, 75% und 125% des individuellen Energiebedarfs mit 50% Kohlenhydrate, 35% Fett, 15% Protein). Die verschiedenen EF Niveaus wurden durch Laufen (4 km/h) auf einem Laufband erreicht (0, 165 and 330 min). Der Energieverbrauch im Schlaf (SEE), die 24-h Makronährstoffoxidation und die relative Makronährstoffbilanz (Oxidation relativ zur Aufnahme) wurden bestimmt.

Während EB und OF stieg die 24-h Fettoxidation mit höherem EF an. Das resultierte in einer höheren relativen Fettbilanz mit mittlerem EF (EB: +17%, OF: +14%) and hohem EF (EB: +23 %, OF: +17%) verglichen mit niedrigem EF (alle $p < 0.05$). Der SEE während EB und OF war höher mit mittlerem (EB: +5 kcal/3h and OF: +12 kcal/3h) und hohem (EB: +7 kcal/3h and OF: +18 kcal/3h) EF verglichen mit niedrigem EF (alle $p < 0.05$). Während CR war, dagegen die 24-h Fettoxidation lediglich bei hohem EF höher verglichen mit niedrigem EF und weder die relative Fettbilanz noch der SEE unterschieden sich zwischen den EF Niveaus.

Ein höherer EF könnte sich günstig auf die Körpergewichtsregulation während kurzfristiger Phasen der Überernährung und bei ausgeglichener Energiebilanz auswirken. Bei einer kurzfristigen Kalorienrestriktion zeigte ein höherer EF jedoch keinen Einfluss auf die Regulation der Energie- und Fettbilanz. Ein höherer EF kann daher vor allem die negativen Auswirkungen einer kurzfristigen Überernährung abschwächen.

Zusammenfassend zeigt diese Arbeit die hohe Bedeutung von körperlicher Aktivität im Alltag. Ein hoher *Energy-Flux* könnte negativen Auswirkungen auf den Glukose-stoffwechsel, die durch das Auslassen des Frühstücks zustande kommen, möglicherweise abschwächen.

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Abbreviations

$\dot{V}CO_2$	carbon dioxide production
$\dot{V}H_2O$	water vapor production
$\dot{V}O_2$	oxygen consumption
(FAT)/CD36	fatty acid translocase
AEE	activity energy expenditure
AMPK	AMP-activated protein kinase
ATP	adenosine triphosphate
BP	barometric pressure
BSD	breakfast skipping day
CHO_i	carbohydrate intake
CHO_{ox}	carbohydrate oxidation
CPT-1	carnitine palmitoyltransferase 1
CR	caloric restriction
DIT	diet-induced thermogenesis
DSD	dinner skipping day
EB	energy balance
ECG	electrocardiographic recording
EE	energy expenditure
EE_0	energy expenditure in the inactive state
EE_{active}	energy expenditure during activity
$EE_{inactive}$	energy expenditure during inactivity
EF	energy flux
E_i	energy intake
EPOC	excess post-exercise oxygen consumption
fCO_{2e}	fractional concentration of carbon dioxide in the excurrent airstream
fCO_{2i}	fractional concentration of carbon dioxide in the incurrent airstream
FFA	free fatty acids
fH_2O_e	fractional concentration of water vapor in the excurrent airstream
fH_2O_i	fractional concentration of water vapor in the incurrent airstream
F_i	fat intake
FMI	fat mass index

fO _{2e}	fractional concentration of oxygen in the excurrent airstream
fO _{2i}	fractional concentration of oxygen in the incurrent airstream
F _{ox}	fat oxidation
FR	flow rate
FR _e	flow rate of the excurrent airstream
FR _i	flow rate of incurrent airstream
HOMA-IR	homeostasis model assessment-insulin resistance
HOMApp	postprandial homeostasis model assessment
HRV	heart rate variability
iAUC	incremental area under the curve
MAGE	mean amplitude of glycemic excursions
npRQ	non-protein respiratory quotient
O ₂	oxygen
OF	overfeeding
PAL	physical activity level
P _i	protein intake
P _{ox}	protein oxidation
rCHO _B	relative carbohydrate balance
rE _B	relative energy balance
REE	resting energy expenditure
rF _B	relative fat balance
RMSSD	root-mean-square differences in successive normal-to-normal intervals
rP _B	relative protein balance
RQ	respiratory quotient
SDNN	standard deviation of all normal-to-normal intervals
SEE	sleeping energy expenditure
SNS	sympathetic nervous system
tAUC	total area under the curve
TEE	total energy expenditure
VO _{2max}	maximal oxygen uptake
WVP	water vapor pressure
ΔO ₂	difference between incurrent and excurrent O ₂

Chapter I

Introduction

Perfect regulation of energy balance in order to maintain body weight is not only a matter of energy intake on the one hand and energy expenditure on the other but rather of a dynamic interdependence between them [1]. Diet and physical activity are the main players that modulate this dynamic energy balance equation and should be understood as synergistic and strongly interlinked [2]. To combat the obesity epidemic, it is, therefore, necessary to focus on both players at the same time [3]. Daily energy expenditure is influenced by total energy intake [4], but energy intake is affected by physical activity [5] and body mass, which are the major determinants of energy expenditure [6].

In general, there is a big confusion in the population about body weight management and too often one dietary trend supersedes the other without conclusive scientific evidence. Most of the time these dietary trends are simply based on caloric restriction [7]. For example, in recent years skipping meals in order to prolong fasting periods becomes popular and is frequently advertised [8], although epidemiological studies suggest that skipping breakfast is a risk factor for overweight [9,10], type 2 diabetes [11] and

cardiovascular health [12]. Therefore, it is necessary to investigate meal skipping independent of a negative energy balance under well-controlled energy balance conditions.

Besides this, everyday physical activity declined massively in the last 150 years and a sedentary lifestyle became pervasive [13]. This everyday physical activity can be classified as very low intense physical activity and includes making the way to work, be active at the job, housekeeping, gardening etc. as opposed to exercise physical activity [13]. Since energy expenditure can vary among individuals of the same size by 2000 kcal/d, mainly due to non-exercise activity energy expenditure [13], this reduction of physical activity might have a huge impact on the regulation of energy balance. Human physiology developed under conditions, where energy balance was reached at a high level of physical activity (high energy flux = high energy intake matches high energy expenditure) [3]. This suggests, that a person with low energy throughput is at risk of gaining weight [3].

From early research on energy expenditure, it is known that a low resting energy expenditure (REE) in relation to body size [14,15] and a low fat oxidation (assessed as a high respiratory quotient) [16,17] is a predictor for long-term weight gain. These findings were later confirmed [18,19] and it was shown, that they are influenced by dietary patterns [20,21] and physical activity [22]. Although, it is unclear if an impaired metabolism follows weight gain or promotes weight gain. In long-term studies, the assessment of energy balance is accomplished indirectly by assessment of changes in body weight or body composition [3]. Thus, to improve the understanding of the dynamic relationship between energy intake and energy expenditure on metabolic risk, it is necessary to also investigate the short-time regulation of acute energy and nutrient balance.

Therefore, this thesis aimed to investigate the short-term impact of energy flux and meal skipping on energy- and macronutrient metabolism using a metabolic chamber and tightly controlled protocols of nutrition and physical activity interventions.

Using metabolic chambers to study energy and macronutrient metabolism by indirect calorimetry

Since the first law of thermodynamics is valid for the human body, the amount of heat dissipated to the environment can be measured and considered for the assessment of energy expenditure [23,24]. This method of direct measurement of heat loss has been used since the end of the eighteenth century [24]. However, direct calorimetry for the assessment of energy metabolism is strongly limited, not at least because of large response time in regard to changes in energy expenditure due to heat storage within the body. In contrast, indirect calorimetry, which is based on the heat released by oxidative processes, is able to overcome this disadvantage [23]. Using indirect calorimetry, as the name suggests, it is possible to indirectly calculate heat released by chemical processes by measuring the rate of oxygen consumption ($\dot{V}O_2$) [24]. It was shown in a resting subject that the amount of heat release by oxidative processes and the amount of heat loss to the environment are identical, demonstrating the concept of indirect calorimetry [25]. All energy dependent processes in the human body utilize the energy released by adenosine triphosphate (ATP). The generation of ATP is an oxygen consuming step with a proportional relation between $\dot{V}O_2$ and ATP synthesis [26]. Due to the constant arterio-venous difference of oxygen (O_2) in the blood, the rate of O_2 in the exhaled air is a direct reflection of $\dot{V}O_2$ of the tissue [24]. To soundly calculate energy expenditure, besides $\dot{V}O_2$, it is necessary to measure carbon dioxide production ($\dot{V}CO_2$) of the subject and urinary nitrogen excretion. Because of the nature of the fuel mixture oxidized, the energy equivalent of oxygen is not constant but depends on the type of nutrients oxidized [23]. Measuring these three parameters makes it possible to calculate fat, carbohydrate and protein oxidation and thus not only allows to study energy expenditure, but also macronutrient oxidation [27].

The technique of indirect calorimetry can be applied using diverse systems allowing the assessment of different aspects of energy expenditure and macronutrient oxidation [28]. For only short-term measurements of respiratory gases, metabolic carts like ventilated hoods or facemasks are suitable because they have a fast response time [29]. Metabolic carts are relatively easy in handling and used in most clinical research settings, though measurements are limited to a few hours [29]. To measure energy expenditure

and macronutrient oxidation continuously for longer time periods (up to several days), in approximate free-living conditions, metabolic chambers are unique. In particular, they offer the opportunity to assess acute energy and nutrient balances [30,31]. In Addition, components of energy expenditure like sleeping metabolic rate, diet-induced thermogenesis, and activity energy expenditure can be measured over a prolonged period [28]. Modern metabolic chambers are whole-room indirect calorimeters, where respiratory gases of an inhabited subject are assessed by measuring the changing composition of the air contained in the metabolic chamber [32]. The first metabolic chambers were developed by Pettenkofer in 1862 [33] and Atwater in 1904 [34]. Since then, great progress has been made in measuring instruments, data acquisition software and data processing [32,35–38]. At first, metabolic chambers were only used to assess long-term parameters (24h-measurements) rather than rapid changes, because the large size of the room constituted a significant problem in terms of the response time [38]. However, due to improvements, metabolic chambers can today be used to study dynamic changes in energy metabolism [31] and the short-term kinetics of substrate oxidation [39]. They are considered as the current gold standard for assessing minute to minute changes in energy expenditure [32]. The growing importance of metabolic chambers in the research of energy metabolism is reflected by the increasing number of metabolic chambers from 2-3 in the early 1980s to > 30 nowadays worldwide [31].

Nevertheless, the accurate measurement of human metabolism by metabolic chambers is still challenging and requires methodological knowledge. Therefore, in this thesis, critical issues that were faced implementing two metabolic chambers are presented. Important basic principles are summarized to promote a better understanding and the method of whole-room indirect calorimetry is discussed.

Impact of meal skipping on body weight regulation

Eating in misalignment with the biological clock, e.g. skipping breakfast and eating bigger meals in the evening or eating late at night is associated with increased risk for obesity and type 2 diabetes [40,41]. On the other hand, popular trends like breakfast skipping or dinner skipping are advertised for weight management. Conclusive scientific evidence to support these suppositions is however lacking [8].

Previous tightly controlled room calorimetry studies that have investigated the impact of meal frequency on regulation of energy balance under isocaloric conditions did not find a difference in energy expenditure between large (1-2 meals/d), normal (3 meals/d), or small frequent (> 5 meals/d) patterns [20,21,42–44]. Although no effect of the feeding frequency on mean 24-hour energy expenditure and the respiratory quotient (RQ) was observed in these studies, a lower frequency of 2 or 3 compared with 6-14 meals increased sleeping or resting metabolic rate [20,21], diet-induced thermogenesis (DIT) [45] and changed the diurnal pattern of nutrient partitioning to increased fat oxidation until noon with breakfast skipping [44,45]. The timing of meal consumption has also been shown to affect DIT with higher levels in the morning than in the afternoon and night [46]. A 44% lower DIT in the evening than in the morning [47] argues against a beneficial effect of breakfast skipping on energy balance and rather suggests a favorable impact of dinner skipping. Therefore, it was hypothesized that breakfast skipping compared with dinner skipping leads to lower total energy expenditure.

Diurnal differences in energy expenditure and nutrient partitioning can be mediated by sympathetic nervous system (SNS) activity and endocrine factors. With lower meal frequency, higher peaks and subsequently lower troughs of insulin might lead to increased fat oxidation [21]. In addition to meal frequency, circadian rhythms in insulin sensitivity are known to affect blood glucose levels and insulin secretion in response to meal timing. Thus, the same meal consumed in the evening not only leads to a lower metabolic rate but also increases glycemic and insulinemic responses, suggesting circadian variations in energy expenditure as well as the metabolic pattern of healthy individuals [48]. A nocturnal lifestyle with breakfast skipping and a delayed eating pattern thus can lead to increased 24-h glycemia [44] and impairment of insulin response to

glucose [40] and could, therefore, contribute to an increased risk of type 2 diabetes. It was, therefore, hypothesized that breakfast skipping compared to dinner skipping leads to impaired glucose metabolism.

The primary aim of this study (*Chapter III*) was to compare the effects of breakfast skipping vs. dinner skipping on 24-h energy expenditure and substrate partitioning, as well as (secondary aims) on 24-h SNS activity, insulin-, glucose- and appetite profiles by using a 3-meal control day as a reference and applying well-controlled energy balance conditions in a metabolic chamber.

Impact of energy flux on body weight regulation

According to the concept of energy balance, energy intake must equal energy expenditure to maintain body weight [3]. Thus, to lose body weight preferably in the form of body fat, caloric restriction diets are usually performed [49]. However, a recent observational study by Hume et al. suggests that increasing energy expenditure rather than caloric restriction is more effective for reducing body fat [50]. This study builds on the theory of an asymmetric body weight regulation proposed by Mayer et al. in 1956. This theory is based on the investigation of dietary intake of 213 workers in West Bengal who showed large differences in their daily physical activity [51]. It was observed that the energy intake increased with increasing activity. However, this linear relationship between energy intake and energy expenditure was observed only in a certain range of physical activity. Below this range, a decrease in activity did not lead to a decrease in food intake. In contrast, an increase in food intake was noted and body weight was also increased below this activity threshold. Thus, this theory describes that the regulation of body weight is more effective when energy flux (EF) is high. EF describes the level of energy balance. A high EF can be reached, when energy expenditure and corresponding energy intake, are both high. Accordingly, a low EF describes a sedentary state with low energy expenditure coupled with low energy intake [52]. The observation of a lack of decreasing energy intake when physical activity energy expenditure is low has been confirmed by a recent intervention study by Stubbs et al. [5].

Together with increased food intake, a decreased energy expenditure due to a sedentary lifestyle [13], is viewed as the main cause of obesity [1]. Therefore, the impact of EF on energy expenditure must be considered to further investigate the theory on an asymmetric regulation of body weight.

Hume et al. proposed that a high EF prevents long-term fat gain because of its association with an increased resting metabolic rate [50]. In two interventional studies, an increased REE was observed during conditions of a high EF compared to a low EF [22,53]. These studies have in common that the high EF condition was achieved by exercise with medium to high intensities (50-75% maximal oxygen uptake, VO_2max). Bullough et al. investigated trained and untrained participants and further suggested that the acute effect rather than a training effect of exercise influences the increase in REE [22]. Bell et al. investigated the effects of a reduction in EF in habitually exercising older adults and observed a decreased REE, which has been explained by the reduction of skeletal muscle SNS activity [52]. Therefore, it remains unclear whether physical activity of low intensity, which corresponds to everyday physical activity, is able to increase REE under conditions of a high EF.

Besides energy balance, fat balance is crucial in the obesity issue [54]. A study comparing participants with different levels of fitness observed that those with medium and high fitness levels had better fat utilization (lower RQ) than participants with low fitness levels [55]. Long-term studies on weight loss observed that study groups with combined exercise and caloric restriction have greater fat loss than groups treated with diet-induced weight loss alone and even study groups assigned to exercise without caloric restriction had similar fat mass reductions without changes in body weight than those in the diet-induced weight loss group [56,57]. However, there was no impact of physical activity (≤ 60 min exercise/day ≈ 400 -530 kcal) with low (40% VO_2max), medium (50% VO_2max) or high (70% VO_2max) intensity on 24-h fat oxidation during energy balance measured in a metabolic chamber [58–60]. It is well known that physical activity of low intensity, increases the contribution of fat oxidation to total energy expenditure (TEE) as compared to physical activity of high intensity [61,62]. The effects of prolonged physical activity (> 60 min/d) with low intensity on 24-h fat oxidation, fat utilization and on the acute regulation of fat balance have not yet been studied. It remains unclear whether

there is a dose-response relationship between physical activity and these metabolic outcomes.

In order to investigate the underlying mechanisms of energy balance regulation, it is important to understand that situations of equal energy balance are rare in real life [63]. The concept of energy balance implies that long-term body weight stability is achieved when over time energy intake matches energy expenditure [3]. However, this approach disregards that based on a day to day basis or even shorter time periods, there is a continuous change between overfeeding for example at weekends or holidays and subsequent compensatory underfeeding [64,65]. Therefore, it is in particular of interest to investigate the impact of EF during such short-term over- and underfeeding periods. The effectiveness of energy balance regulation can only be measured when energy balance is challenged due to caloric restriction and overfeeding.

During caloric restriction, resting metabolic rate was shown to decrease [66–70]. Martin et al. observed a 91 kcal/d lower REE adjusted for fat-free mass after 6 months of a 25% caloric restriction diet [66]. More recently, in a study of Schlögel et al. a larger decrease of TEE in response to 24-h fasting in a metabolic chamber correlated with weight gain [71]. This decrease in energy expenditure, independent from fat-free mass is described as adaptive thermogenesis in response to caloric restriction [72] and can enhance the risk of a positive energy balance during weight maintenance after weight loss [3,73]. Based on the assumption of a higher REE with increased EF, it can be hypothesized, that a high EF might counteract this metabolic adaptation to caloric restriction.

In the case of overfeeding it is discussed if adaptive thermogenesis in form of excessive energy dissipation exists [74]. There are some studies, which found an increased energy expenditure with overfeeding [75–77], but the results of several other controlled studies questioned the existence of overfeeding induced energy dissipation [78–83]. However, energy dissipation might appear in the activity related energy expenditure components [69]. Thus, a high EF during overfeeding might improve the regulation of energy balance by promoting energy dissipation. Further, it can be hypothesized that a higher EF prevents the attenuation of fat oxidation due to overfeeding.

The aim of this thesis was to investigate the impact of acute variations in EF reached by different levels of physical activity of low intensity on energy expenditure (primarily sleeping energy expenditure) and regulation of fat balance (fat oxidation as a percentage of fat intake and energy expenditure) during short-term energy balance, caloric restriction (-25% of energy requirement) and overfeeding (+25% of energy requirement). Therefore, a tightly controlled intervention study in a metabolic chamber was performed (*Chapter IV*) with three different EF levels.

Objectives

This thesis investigates the impact of meal skipping and energy flux on the acute regulation of energy and macronutrient metabolism in a metabolic chamber by addressing the following objectives.

Chapter II

- (i) Identification of critical issues implementing a metabolic chamber system
- (ii) Development of solutions for existing problems, that occurred during the measurements

Chapter III

Main hypothesis: Breakfast skipping has an adverse impact on the regulation of energy balance and metabolic health compared to dinner skipping

- (i) Impact of meal timing modulated by skipping breakfast vs. dinner on regulation of energy balance and metabolic risk
- (ii) Impact of meal frequency modulated by skipping breakfast or dinner compared to a conventional three-meal-structure on regulation of energy balance and metabolic risk

Chapter IV

Main hypothesis: The regulation of energy balance and fat balance is improved with higher energy flux

- (i) Impact of energy flux on the regulation of energy and macronutrient balance (e.g. by affecting sleeping energy expenditure and fat oxidation) during energy balance, caloric restriction and overfeeding
- (ii) The ability of an increased energy flux to prevent a decrease in energy expenditure with caloric restriction and to promote energy dissipation during overfeeding
- (iii) The ability of an increased energy flux to prevent a decrease in fat oxidation with overfeeding or to further promote fat oxidation during caloric restriction

Chapter II

Implementation of a metabolic chamber system

Two metabolic chambers have been implemented at the Institute of Nutritional Medicine of the University of Hohenheim to study the regulation of energy and macronutrient metabolism. They are 9 m² rooms and are furnished with a daybed, chair, and desk, computer with internet access, telephone, toilette and washbowl (**Figure 1**). The metabolic chambers have an external window and a semi-transparent perspex door. Daylight can thus promote a cozy atmosphere.

The following chapter describes important principles of metabolic chamber measurements, the specifics of the system and critical issues that were faced when implementing the system. In addition, the way from raw data acquisition to the calculation of energy expenditure and macronutrient oxidation is presented.



Figure 1 Pull-type metabolic chamber based on open-circuit indirect calorimetry located at the University of Hohenheim, Institute of Nutritional Medicine. Right photograph shows the exterior view of the metabolic chamber. Left photograph shows the interior of the metabolic chamber, which includes a daybed, chair and desk, computer with internet access, telephone, toilette, and washbowl.

Important principles of flow-through respirometry applied to a chamber system and its implementation in Hohenheim

There are two main flow-through respirometry methodologies, the push and the pull mode respirometry, whereby push and pull relate to the air flow that is either pushed into the chamber or is pulled from the chamber [84]. Both systems have in common, that the change in gas concentration surrounding the subject, is measured to calculate energy expenditure and macronutrient oxidation. Therefore, it is necessary to measure oxygen and carbon dioxide concentrations in the air before (incurrent) and after (excurrent) exposure to the subject [84]. Both systems have advantages and disadvantages.

In a push-mode chamber, the incurrent air can be easier controlled and maintained stable and the lag-time between chamber and gas analyzer is smaller than in a pull mode system [37]. A push-mode system is mainly used in chambers for small animals because with increasing chamber size the probability for leaks, which are difficult to prevent and hard to find, becomes higher. A push mode system has limitations because all expired air has to be collected to obtain accurate results. In case of any leak, the chamber air can escape to the environment without getting analyzed [84]. In Hohenheim, a pull-system was implemented. This respirometry system is the prevailing technique in today's room

calorimetry [30,32,35,36,85–87]. Even when the chamber is not perfectly sealed, the chamber air does not escape, since it is pulled from the chamber [84]. A slight overpressure in the area surrounding the metabolic chamber can further ensure that all expired air is leaving the chamber through the excurrent flow port [37]. One critical point with pull-type systems is to maintain a reasonably stable composition of the gas flowing into the chamber (incurrent gas concentrations). Reaching a stable incurrent gas concentration can be facilitated by placing the chamber in an isolated room [37].

Determination of the flow rate

Calculation of gas concentration requires that the flow rate (FR, l/min), by which air is pushed or pulled through the system is known. The following four equations represent the underlying principles for the respirometry equations based on the assumption that the nitrogen concentration is not affected by the subject and is therefore identical in the incurrent and excurrent air stream [37]:

$$FRe = FRi - \dot{V}O_2 + \dot{V}CO_2 + \dot{V}H_2O$$

$$\dot{V}O_2 = FRi \times fO_{2i} - FRe \times fO_{2e}$$

$$\dot{V}CO_2 = FRe \times fCO_{2e} - FRi \times fCO_{2i}$$

$$\dot{V}H_2O = FRe \times fH_{2Oe} - FRi \times fH_{2Oi}$$

where FRe and FRi are the FRs of the excurrent and incurrent airstreams; fO_{2i} , fCO_{2i} , fH_{2Oi} and fO_{2e} , fCO_{2e} , fH_{2Oe} are the fractional concentrations of the respective gas in the incurrent and excurrent airstreams. $\dot{V}O_2$ is the oxygen consumption; $\dot{V}CO_2$ and $\dot{V}H_2O$ are the carbon dioxide and water vapor productions.

When air flows through the chamber inhabited by a subject, oxygen concentration decreases, carbon dioxide concentration and water vapor pressure increase and temperature changes [37]. Since the flow is volume per time and volume is depending on temperature and pressure, the FR is different on the exhausted side of the system when compared with the incurrent side. Therefore, in order to calculate the changes in gas concentrations, it is necessary to consider whether flow metering is done at the incurrent

or the excurrent side of the system. Depending on whether the incurrent or the excurrent FR is known, different respirometry equations must be used for the calculations [37].

In general, there are three factors that are required to calculate the FR: the body mass of the subject, the quality of the equipment and the desired temporal resolution. The body mass is needed to predict the subjects $\dot{V}O_2$ [37]. After choosing an appropriate value for the difference between incurrent and excurrent O_2 (ΔO_2), FR can be calculated using standard respirometry equations as $FR = \frac{\dot{V}O_2}{\Delta O_2}$ [37]. A reasonable FR should cause an O_2 -depletion or CO_2 -enrichment between 0.001-1%. The higher the FR the greater the risk to meet the noise and drift floor of the gas analyzers (depending on equipment quality). Thus, the generated FR should typically yield an O_2 -depletion between 0.1-0.5% in order to minimize the effect of analyzer drift on the low side and hypoxia on the high depletion side [84].

If the subjects' predicted $\dot{V}O_2$ is 0.30 l/min and depletion of O_2 is 0.1% on the low side and 0.5% on the high side:

$$FR = 300 \text{ l/min } (= \frac{0.30 \text{ l} \times \text{min}^{-1}}{0.001}) \text{ and } 60 \text{ l/min } (= \frac{0.30 \text{ l} \times \text{min}^{-1}}{0.005})$$

Thus, in this case, FR should be between 60 l/min and 300 l/min. A typical FR used for room calorimetry with a room size of about 20.000 liters is 80 l/min. Based on these two factors a so-called time constant or response time can be calculated. This response time refers to the time that is required for a step change in respiratory gas exchange to reach 63% ($= 1 - \frac{1}{e}$) of its final value within the chamber (Lighton 2008). In the case of monitoring short-term activities, the FR can be increased to reduce response time and maximize temporal resolution. However, increasing FR is always accompanied with increased ΔO_2 measurement error and is limited depending on the quality of respiratory equipment because a high FR decreases the gas concentration changes in the chamber [37].

Correction for water vapor dilution

Besides FR and the oxygen and carbon dioxide concentrations of the incurrent and excurrent air stream, it is necessary to know the water vapor pressure (WVP) in the excurrent air. Because of interactions between the various gas species in the air stream, an increase in water vapor results in a dilution of O₂ and CO₂ [88]. In the case of O₂, the O₂ consumption of the subject in the metabolic chamber will be overestimated if the dilution effect, caused by increased WVP, is not taken into account.

There are two distinct ways to compensate for the dilution effect of water vapor. Either the air stream can be dried by a desiccant to remove water vapor or the water vapor dilution effect can be corrected mathematically. The first option has disadvantages because of the possible incomplete removal of water vapor and the need for toxic desiccants. The latter option requires additional analyzers for WVP and barometric pressure (BP) [37]. Dilution is proportional to WVP's contribution to overall pressure. Thus in order to compensate for water vapor dilution the following equation describes the correction in the case of O₂: $O_2' = \frac{O_2 \times BP}{(BP - WVP)}$ [84].

If the FR is measured at the excurrent side of the system, it is also necessary to correct the FR for water vapor. Otherwise, FR is overestimated compared to its dry state. In this case, the correction is the inverse of water vapor dilution correction ($FR' = FR \times \frac{(BP - WVP)}{BP}$) [37,84].

Baselining

Baselining describes measuring incurrent gas concentrations of the flow-through respirometry [84]. Since the differences in gas concentrations between incurrent and excurrent air are the basis for the calculation of metabolic rate, baselining is crucial for accurate respirometry [84]. In general, the baseline can be measured by switching the analytical gas stream between incurrent and excurrent flow. This kind of baselining cause a disruption of the excurrent trace, while the incurrent trace is measured, which leads to difficulties in large metabolic chambers when response time correction (see *Acquisition and processing of raw data*) is required [37]. Therefore, many calorimeter systems are

working with the assumption of constant incurrent gas concentrations [88]. This problem can be solved by an approach known as "background baselining", for which the company Sable Systems (Sable Systems International, Las Vegas, USA) applied for a patent. Background baselining permits constant and uninterrupted measurement of the excurrent air stream from the chamber while allowing frequent measurement of the incurrent air stream flowing into the chamber. This is accomplished by a twofold design that contains two separate gas analyzer chains each with O₂, CO₂, WVP, and BP analyzers. Both analyzers monitor the excurrent air from the metabolic chambers and at regular intervals, the input of one analyzer chain is switched to measure the incurrent air. Thereby, never both analyzers at the same time switch to measure the incurrent air, but they alternate between each other with the measurement of incurrent air [37].

During background baselining, the oxygen analyzer drift is also corrected with the incoming air. Oxygen analyzer drift can be a major source of error in room calorimeters, due to the long duration of the measurements [88]. Because O₂-concentration in the atmosphere is very stable and it can be assumed, that the fractional concentration of dry, CO₂-free air is 0.2094 [89], the O₂-traces can be spanned to this known incurrent concentration in order to correct for analyzer drift [37].

Specification of the implemented metabolic chamber system

The implemented metabolic chambers at the University of Hohenheim are two pull-type metabolic chambers with a volume of 21,000 liters (D&S Consulting Services Inc., New York, NY). Airlocks are used for the exchange of food and equipment (Life Science Technologies International LSTi, Leun, Germany). The airtight door of the chambers (Life Science Technologies International LSTi, Leun, Germany) has an examination hatch, which makes it possible to take blood samples of the participants without opening the door. In case of an emergency, the door can be opened from inside by pressing a button. For continuously mixing and tempering the room air, the chambers are equipped with an air conditioner (Mitsubishi Electric MSZ-SF15VA, Tokio, Japan). Rates of oxygen and carbon dioxide concentrations are measured continuously using the Promethion integrated whole room indirect calorimeter system (Sable Systems International, Las Vegas, USA).

The system consists of a GA-3m2 gas analyzer and an FG-250 flow generator. Oxygen and CO₂-concentrations (%), as well as WVP and BP, are measured by two distinguished gas analyzer chains, allowing background baselining as described above (see *Baselining*).

A chamber volume of 21,000 liters and a typical FR of 80 l/min resulting in a time constant of 262.5 minutes. This means that it takes over 4 h to replace the total chamber volume. Therefore, to observe any short-term changes of metabolic rate, a mathematical correction for response time is required.

To measure O₂ and CO₂ to an accuracy of 0.001% a galvanized fuel cell analyzer (Maxtec, Salt Lake City, USA) and a non-dispersive infrared analyzer (Sable Systems International, Las Vegas, USA) are used. WVP of the sample gas stream is measured directly to 0.001 kilopascals (kpa) by a capacitive humidity sensor and the results are utilized to continuously correct the $\dot{V}O_2$ and $\dot{V}CO_2$, along with mass air flow (L). Since all analyzers drift, it is important especially for measurements over 24 h to compensate for analyzer drift during the experiment. This is achieved during frequent background baselining as described above. In addition, to avoid that the individual analyzers of the twofold design drift differently, they are installed in one case and the temperature in this analyzer unit is stabilized to 37°C.

Fresh air is drawn from the environment above the building and flows into the chambers from an area behind the chambers. This area will not be entered, acting as a buffer. A sound and unexhausted baseline without fast fluctuations can thus be ensured. Baseline samples (incurrent air) are obtained from right above the inflow of this fresh air to the metabolic chambers. In consideration of a time constant between two to four h, depending on the FR, it is hard to compensate for rapid changes in the incurrent air even when it is measured, making a stable incurrent air very important. In Addition, a slight positive air pressure in the room surrounding the two metabolic chambers guarantees that there is no air leaking of the metabolic chambers.

Acquisition and processing of raw data

In order to conduct a metabolic chamber measurement, the software CaloScreen v1.3.5/v1.3.10 (Sable Systems International, Las Vegas, USA) was used. This software enables to configure a recording, to start the measurement and to monitor the acquired raw data in real-time. Setup settings such as FR and baselining-intervals can be configured before the measurement and are performed automatically during the run. Measured values are recorded every second. All raw data, that include the gas concentrations and WVP traces of both analyzer chains as well as the FR, the subsampling FRs and the temperature of the analyzer unit, are saved to a file for subsequent data processing.

Further data processing is performed by using Sable Systems ExpeData software (version 1.7.31 and 1.9.51). This software uses a specific macro code that performs all the data analyses. The macro encodes signal preconditioning, a WVP dilution correction, a baseline drift correction from the incurrent measurement and the calculation for mathematically derived dry FR. The incurrent O₂-baselines of the two channels are spanned to 0.2094 in order to eliminate analyzer drift using a so-called “Catmull-Rom splining technique” (local interpolating spline functions between control points to approximate a curve) [90]. In addition, the traces of O₂ and CO₂ of the two analyzer channels are merged by averaging the values and a single delta O₂ and CO₂ channel is created. $\dot{V}O_2$ and $\dot{V}CO_2$ were calculated using the following equations, originated by Brown et al. (1984):

$$\dot{V}O_2 = FR \times \frac{(O_2 - 0.2094 \times CO_2)}{(1 - 0.2094)}$$

$$\dot{V}CO_2 = FR \times \frac{(CO_2 + FiCO_2 \times O_2)}{(1 + FiCO_2)}$$

As mentioned above, a time constant of at least two hours requires a response time correction. Because the expired air of the participant in the metabolic chamber is distributed in the chamber and mixed with incurrent air before it is pulled from the chamber and flows to the gas analyzers, changes in $\dot{V}O_2$ and $\dot{V}CO_2$ are not immediately present in the sample air stream [37]. This means that a new level of metabolic rate, e.g. when starting to exercise, can only be determined after a plateau is reached. Therefore, it is necessary that the participant's metabolic rate does not change again during the time

required to reach this plateau. Considering the time constant, it is not possible to calculate changes in metabolic rate without response time correction in human metabolic chambers [37]. Response time correction was performed by z-Transformation, which is based on perfect mixing characteristics of the air in the metabolic chamber and obeys first-order (e^{-kt}) kinetics [84]. In theory, response time correction must consider two parts of response delay. The first part is the time that is required until a gas concentration change in the chamber reaches the analyzer. In other words, the interval between successive samples is needed, which is determined by sampling FR and tubing volume between the chamber and the analyzer. The second part of response delay is the time constant of the chamber. Taken together, a constant z can be determined, describing these characteristics of the system by mathematical analysis of the exponential wash-out curve [84] and it is then feasible to mathematically predict changed $\dot{V}O_2$ and $\dot{V}CO_2$ values [37]. Finally, it follows that the response delay can be corrected using the following equation:

$$VGx(t) = VGy(t) + dVGy(t) \times z$$

where $VGx(t)$ is the z-Transformed final value of $\dot{V}O_2$ or $\dot{V}CO_2$ and $VGy(t)$ is the measured value of $\dot{V}O_2$ or $\dot{V}CO_2$. In words, the original data trace is differentiated and multiplied by the constant z and added to the original data [37].

In practice, the constant z can be determined by conducting a propane burn (see *Validation*) and using ExpeData software to analyze the wash-in curve of the known constant input. By knowing the constant z , response time correction can simply be included in the macro code that is used to process the raw data. In case of the implemented system, a z constant of $1/0.000103$ was used for a FR of 120 l/min and a z constant of $1/0.00008$ was determined for a FR of 80 l/min. **Figure 2** shows the comparison of a z-Transformed (Figure 2B) and not z-Transformed (Figure 2A) dataset. The comparison shows, that with a z-Transformation a new level of metabolic rate or an acute change in metabolic rate is directly reflected in the data trace.

Because of the large value for the response correction a high quality of the data acquisition system is necessary. Since the response correction involves calculation of the derivative of the gas concentration vector and this derivative term is multiplied with this

large constant z , it is extremely sensitive to any noise in the data. Especially, the measurement of O_2 -concentrations can be critical because concentration changes are very small compared to potentially emerging noise (signal to noise ratio) [38]. Response time correction might, therefore, introduce noise to the data, that could overwhelm the metabolic signal [37]. Data processing techniques like the moving average filter, that smooth the data are applied to reduce the noise prior to conduct the response time correction. However, this kind of smoothing the data reduces its temporal resolution and leads to a trade-off between noisy data and temporal resolution of the data in case there is any disturbance in the data acquisition system.

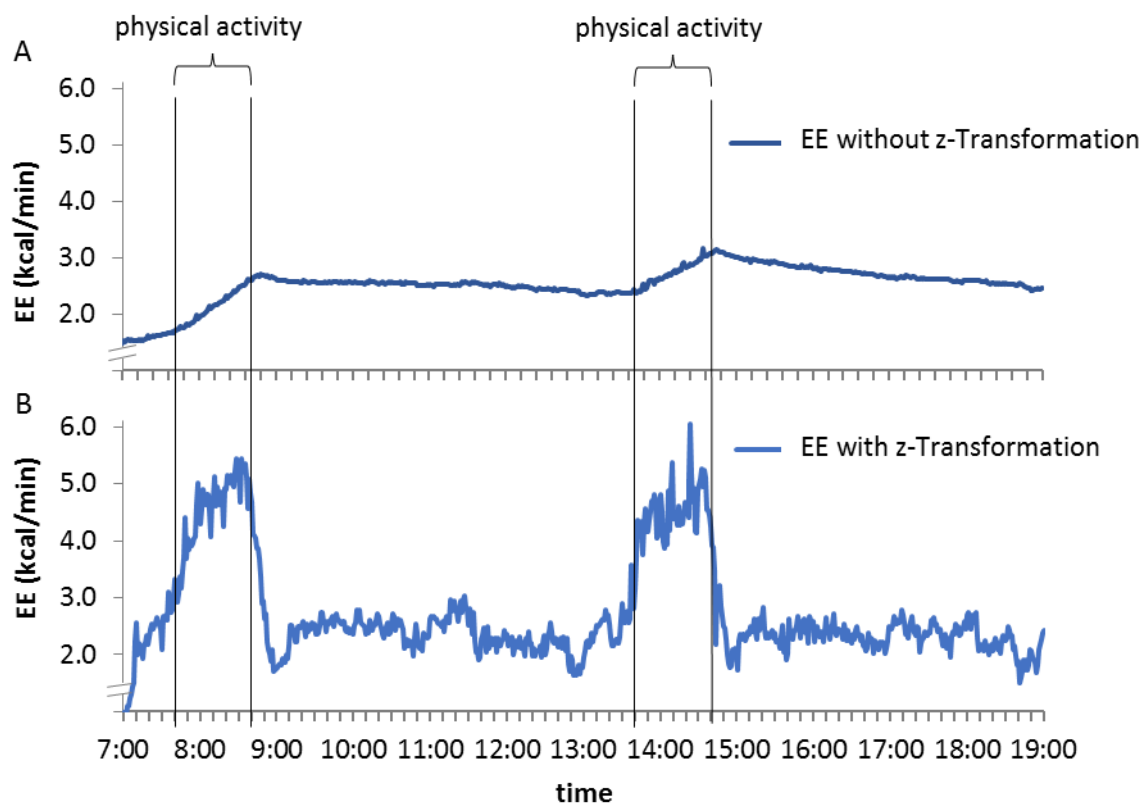


Figure 2 Comparison of a dataset without z-Transformed (A) and with z-Transformed (B) energy expenditure values. The dataset shows an increase in energy expenditure due to physical activity at 0740 and 1340. EE, energy expenditure.

Calibration

During study periods CO₂-sensors were calibrated with nitrogen (CO₂ = zero) and CO₂-span gas (1.0%) approximately every week. O₂-sensors are calibrated with nitrogen once a month. Spanning of the O₂-analyzer is done during data analysis (see background baselining) and before each study test by switching a desiccant column (Drierite, regular (CaSO₄)) into the airstream. Using this desiccant column, WVP analyzers are zeroed by the dried air and spanned by knowing the increase of FiO₂ due to desiccation.

Validation

In order to verify the accuracy of measuring $\dot{V}O_2$, $\dot{V}CO_2$, energy expenditure, and RQ, it is recommended to perform propane burns once a month. Validating the system refers to the recovery rate of $\dot{V}O_2$ and $\dot{V}CO_2$ while burning a known amount of propane in the metabolic chambers. By knowing that 2.542 l of O₂ is consumed and 1.525 l of CO₂ is produced for each gram of propane, the theoretical values can be calculated and compared to the found values of the system. It is expected, that all found variables are > 98% of the calculated values. One drawback of using propane burns to validate metabolic chambers is the problem of incomplete combustion of the propane gas. **Table 1** shows the results of two propane burns conducted with the metabolic chamber of Hohenheim (99.2% propane, Scott Medical Products, Pennsylvania, USA). Propane burn 1 represents a clean burn with a sufficient recovery rate of all variables. Propane burn 2 shows typical results in case of an incomplete combustion of propane.

Table 1 Results of a clean propane burn (1) and a propane burn with incomplete combustion of propane (2) in the metabolic chamber of Hohenheim

	found value	calculated value	recovery rate [%]
Propane burn 1			
$\dot{V}O_2$, liters	141.5	140.5	0.7
$\dot{V}CO_2$, liters	83.5	84.3	-0.9
Energy expenditure, kcal	649.9	657.9	-1
RQ	0.59	0.60	-1.9
Propane burn 2			
$\dot{V}O_2$, liters	89.9	96.3	-6.7
$\dot{V}CO_2$, liters	55.8	57.8	-3.4
Energy expenditure, kcal	415.9	451.2	-7.8
RQ	0.62	0.60	3.1

$\dot{V}O_2$, oxygen consumption; $\dot{V}CO_2$, carbon dioxide production; RQ, respiratory quotient

Assessment of energy expenditure and macronutrient oxidation in a metabolic chamber

Energy expenditure (kcal) and the RQ can be determined continuously from $\dot{V}O_2$ consumption and $\dot{V}CO_2$ production [92].

Total energy expenditure contains three constituents: REE, DIT and activity energy expenditure (AEE). REE is with ~70% the main part of TEE and refers to the energy use for maintaining the biochemical functions of the body at rest [93]. Sleeping energy expenditure (SEE) was shown to be 5% lower than REE [94]. DIT is dependent of the type of macronutrients ingested and the amount of food consumed, hence it refers to the energy required for food processing, i.e. digestion, absorption, assimilation and nutrient storage [29].

The RQ, which is defined as the relationship of produced carbon dioxide to oxygen consumption ($RQ = \frac{\dot{V}CO_2}{\dot{V}O_2}$) provides information about the relationship between fat and carbohydrate oxidation [92]. Based on differences in the chemical structure of the macronutrients, each macronutrient consumes a certain amount of O_2 and produces a certain amount of CO_2 for oxidation [95]. Thus, when glucose is the principal substrate oxidized, the RQ value reaches 1.0 and a value of 0.7 indicates that fat is the main fuel. A mixed oxidation of carbohydrates, fats, and proteins exists when RQ-values are between 0.7 and 1.0 [24].

Using nitrogen excretion in 24-h urine allows a more accurate calculation of energy expenditure [96] and allows to determine a non-protein respiratory quotient (npRQ), which enable the separate assessment of fat, carbohydrate and protein oxidation [92]. Calculation of TEE, SEE, DIT, as well as fat, carbohydrate, and protein oxidation are described in the following two sections.

Assessment of energy expenditure components

Energy expenditure (EE) was determined using the Weir equation [96]:

$$EE \text{ (kcal/min)} = 3.941 \times \dot{V}O_2 + 1.106 \times \dot{V}CO_2 - 2.17 \text{ (kcal/g)} \times \dot{N} \text{ (g/min)}$$

In order to correct for the effect of protein metabolism, nitrogen excretion is used, based on the O_2 equivalent of one gram of urinary nitrogen [27]. Nitrogen was calculated from urinary urea (1g urea contains 46.7% nitrogen), which was measured photometrically from 24-h urine and obligate nitrogen losses by feces and skin were assumed to be +2.5 g \dot{N} /d. Total energy expenditure (kcal/d) was obtained from minute to minute intervals and were calculated from 0600-0600.

SEE was measured as reported by Schrauwen et al. as the lowest energy expenditure value of three consecutive h during sleep between 2400-0600 [97].

DIT was calculated according to Westerterp et al. [98] using a modified version of Schutz's method [99]. This approach computes energy expenditure in the inactive state (EE_0) by plotting physical activity against energy expenditure. The y-intercept of the regression line represents EE_0 (kcal/min) and consists of sleeping metabolic rate and DIT (**Figure 3**). EE_0 is extrapolated over 24-h and DIT can then be calculated by subtracting SEE (extrapolated 24-h values) from EE_0 . For the present study in *Chapter IV*, the regression line of energy expenditure (kcal/h) and physical activity was calculated by plotting 1-h average values over a period of 23-h (0700-0600). Step counts (step count via ActivPAL, Paltechnologies Ltd., Glasgow, UK) were taken as measurand of physical activity. Calculations were performed over 23-h, instead of 24-h because between 0600 and 0700 energy expenditure data of the participants are missing as they left the chamber to place an intravenous catheter.

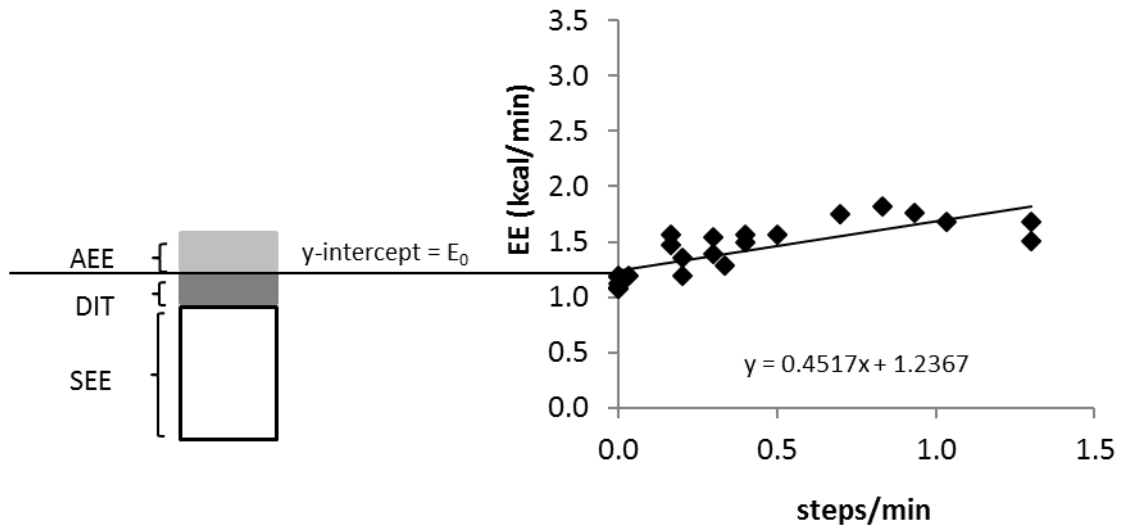


Figure 3 Calculation of diet-induced thermogenesis (DIT) according to Westerterp et al. [98]. Energy expenditure in the inactive state (EE_0) is computed for one subject by plotting physical activity against energy expenditure. The y-intercept of the regression line represents EE_0 (kcal/min) and consists of sleeping energy expenditure and DIT. The approach is modified from Westerterp et al. by using hourly step counts and 60 min averages of energy expenditure over a period of 23-h (each point represents a 60 min average). AEE, activity energy expenditure; DIT, diet-induced thermogenesis; SEE, sleeping energy expenditure; EE_0 , EE in the inactive state; EE, energy expenditure.

Assessment of macronutrient oxidation

Carbohydrate (g/min) and fat oxidation (g/min) are calculated on the basis of the RQ and nitrogen excretion (\dot{N} , in g/min, calculated from urea) using the following equations derived by Jéquier und Felber [24]. First, npRQ needs to be calculated by subtracting the fraction of produced CO_2 and consumed O_2 that refers to protein oxidation:

$$npRQ = \frac{np\dot{V}CO_2}{np\dot{V}O_2}$$

$$np\dot{V}CO_2 \text{ [l/min]} = \dot{V}CO_2 - \text{protein}\dot{V}CO_2 = \dot{V}CO_2 - \dot{N} \times 6.25 \times 0.774$$

$$np\dot{V}O_2 \text{ [l/min]} = \dot{V}O_2 - \text{protein}\dot{V}O_2 = \dot{V}O_2 - \dot{N} \times 6.25 \times 0.966$$

Because protein consists of 16% nitrogen, \dot{N} (g/min) is multiplied with the reciprocal value of 6.25. In order to oxidize one gram of protein, 0.966 l of O_2 is consumed and 0.774 l of CO_2 is produced.

For calculation of the fraction of $np\dot{V}O_2$ derived from glucose oxidation (F_g), the $npRQ$ of a typical fat (palmitoyl-stearoyl-oleoyl-glycerol) is determined with 0.705.

$$F_g = \frac{npRQ - 0.705}{1 - 0.705}$$

Consequently, the fraction of $np\dot{V}O_2$ due to fat oxidation (F_f), is given by the following equation:

$$F_f = 1 - F_g$$

Thus, the rate of glucose oxidation (\dot{g} , g/min) can be calculated by knowing that 1 g of glucose needs 0.746 l O_2 for complete oxidation [27]

$$\dot{g} \text{ (g/min)} = F_g \times \left(\frac{np\dot{V}O_2}{0.746} \right)$$

Note that the oxidation of 1 g fat needs 2.03 l O_2 , the rate of fat oxidation (\dot{f} , g/min) can be calculated as followed:

$$\dot{f} \text{ (g/min)} = F_f \times \left(\frac{np\dot{V}O_2}{2.03} \right)$$

The rate of protein oxidation (\dot{p} , g/min) is represented by $\dot{p} \text{ (g/min)} = \dot{N} \times 6.25$.

Mean values for 24-h macronutrient oxidations were obtained from minute to minute intervals and were calculated from 0600-0600.

For the time periods during which participants left the chamber to place an intravenous catheter, $\dot{V}O_2$ and $\dot{V}CO_2$ were linearly interpolated by a macro code using ExpeData software.

Critical issues implementing the metabolic chambers: The way from noisy data to valid data

Beside all the theoretical considerations, the implemented systems were tested by propane burns for validation (see validation) and by short test runs (not part of this thesis). However, that could not prevent some problems, that were faced during the first both studies that were conducted using the metabolic chambers. These included the occurrence of water vapor fluctuations, problems with the temperature in the gas analyzer units and data processing issues. The following chapter addresses these problems and shows the respective solutions.

Water vapor fluctuations

During the second study that was conducted using the metabolic chambers strange fluctuations in the WVP trace occurred. The onset of these very fast and huge fluctuations was during the summer month and they lead to problems in the data processing procedure. The fluctuations were reflected in the response time corrected traces of $\dot{V}O_2$ and thus lead to fluctuations in RQ and energy expenditure. **Figure 4** shows an example of the WVP fluctuations and corresponding data traces. Figure 4A displays the non-z-Transformed $\dot{V}O_2$ values from one of the two analyzers (VO_2 channel 1). Figure 4B shows the z-Transformed $\dot{V}O_2$ values (VO_{2_c}), Figure 4C and 4D show the z-Transformed RQ and energy expenditure. The Figure shows, that these huge fluctuations caused problems in the corresponding data traces, whereas in case of the occurrence of small fluctuations data processing worked well and the measurement of $\dot{V}O_2$ was not impaired.

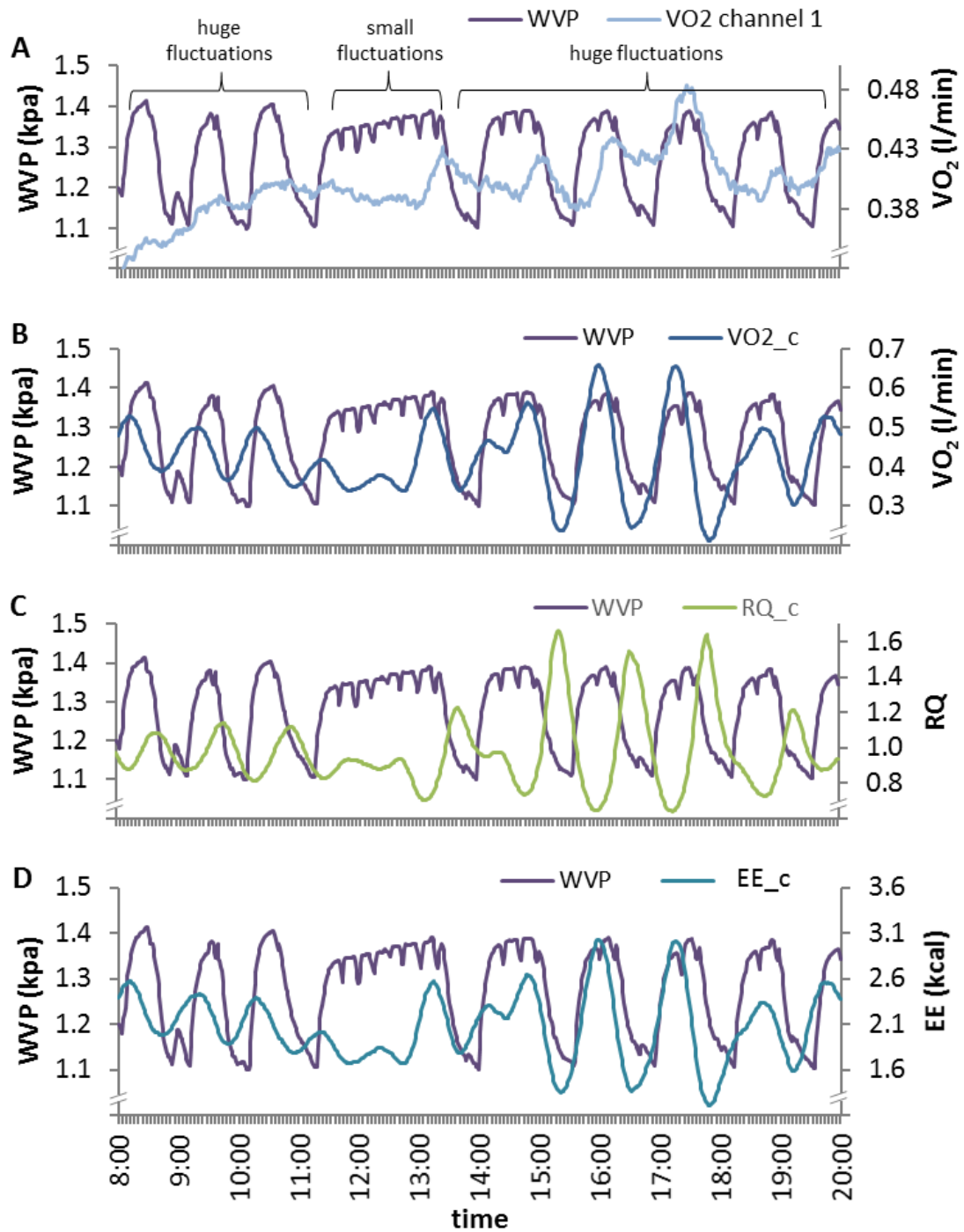


Figure 4 Exemplary dataset of the water vapor pressure (WVP) fluctuations with the corresponding oxygen data trace of one analyzer (VO₂ channel 1) (A), z-Transformed oxygen data trace (VO_{2_c}) (B), respiratory quotient (RQ_c) (C) and energy expenditure (EE_c) (D). The huge fluctuations in the WVP trace were reflected in the response time corrected traces of $\dot{V}O_2$, RQ and energy expenditure.

In order to find the cause of the problem, a test run without a participant was conducted. For this test, baseline intervals and duration were increased while maintaining the other setup settings. Thereby, a disruption from outside the chamber as a causal factor could be excluded because the baseline data (incurrent air) were relatively stable (**Figure 5A**). The test run showed WVP fluctuations in the excurrent WVP trace. Finally, a further test run only with the mixing function but without the cooling function of the air conditioner within the chambers, lead to the disappearance of the water vapor fluctuations in the excurrent WVP trace (**Figure 5B**).

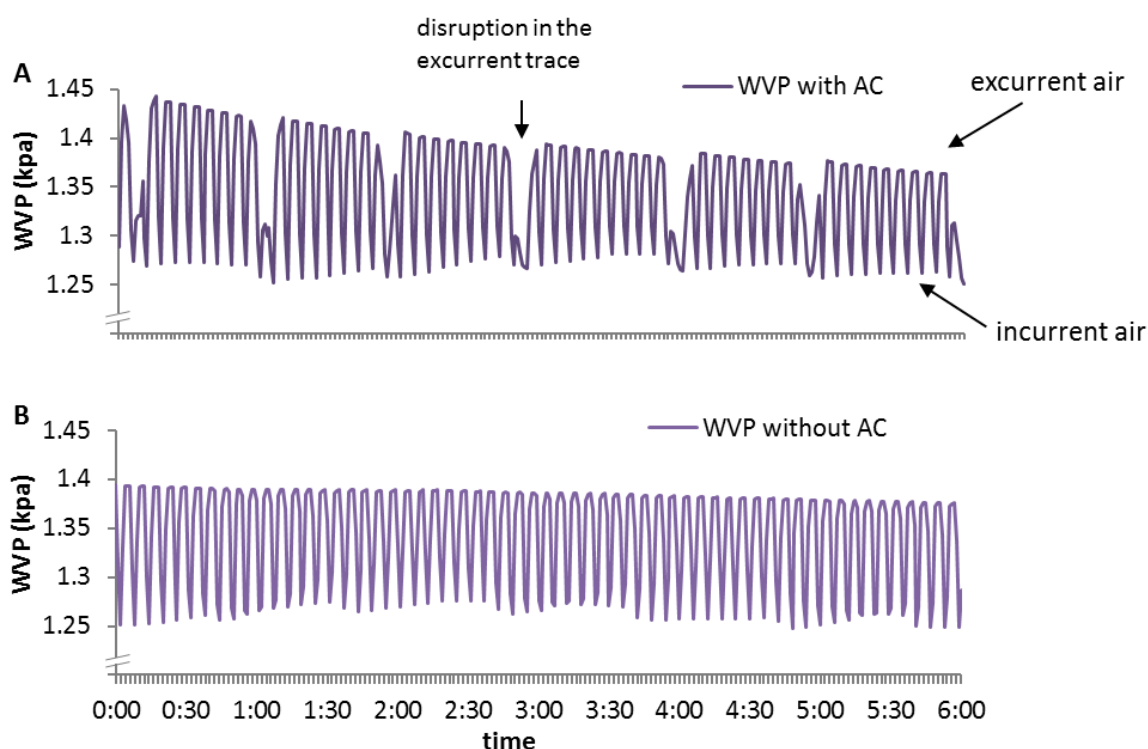


Figure 5 Water vapor pressure (WVP) trace during frequent baselining with air conditioning (A) and without air conditioning (AC) (B). Disruptive fluctuations in the excurrent trace (A) disappeared when the cooling function of the air conditioner was turned off (B).

The air conditioner is cooling the air and simultaneously the air dries automatically because water vapor condenses during cooling, which leads to a decrease of water vapor in the air. The air conditioner works based on a preset temperature, once this temperature is reached in the chamber the air conditioner turns off. Based on the huge WVP fluctuations, the basic principle of operation of the air conditioner, and the high outside temperature, it was presumed that the onset of the cooling function of the air

conditioner was delayed. So, the temperature in the metabolic chamber was already increased far above the preset temperature of the air conditioner, when the cooling function of the air conditioner turned on. In addition, it appeared that the delayed onset of the cooling function was accompanied by an overshooting of the cooling function. Finally, the fluctuations in temperature are reflected in the huge and fast fluctuations of WVP. Unfortunately, the temperature within the chambers was not recorded, thus this data is missing to confirm this assumption.

To solve this issue, the setup of the air conditioner was changed by increasing the preset temperature from 21.5 to 24.5 °C. This was done in order to avoid a fast temperature drop and large cooling gradient in case of an increase in temperature in the metabolic chambers. Increasing the preset temperature lowered the probability that the temperature in the metabolic chambers increased above the temperature threshold and thus was able to avoid the overshooting of the cooling function of the air conditioner. **Figure 6** displays the WVP trace and corresponding $\dot{V}O_2$ data, as well as the RQ and energy expenditure of an exemplary dataset after the setup of the air conditioner was changed. This is, however, a less-than-ideal solution to the problem because the temperature in the metabolic chamber cannot be freely selected. In addition, the relatively warm ambient temperature caused some discomfort for the participants in the chamber especially at night and during physical activity. It would, therefore, be better if the air conditioning system responds faster to temperature changes and keeps the temperature in the metabolic chambers constant within narrow limits. Consequently, it can be assumed that the amplitude of WVP fluctuations is then also small, regardless of which temperature is chosen.

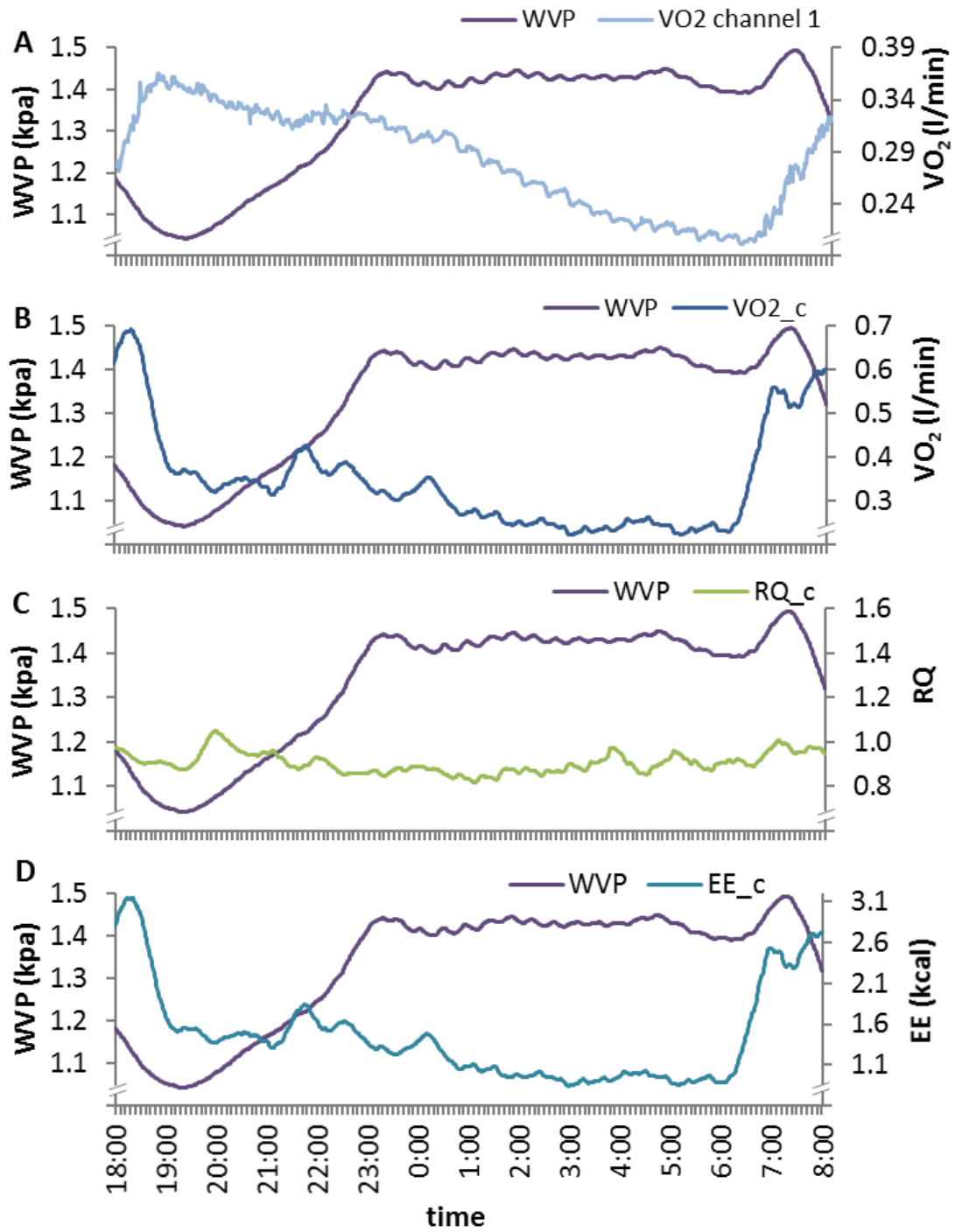


Figure 6 Exemplary dataset of the water vapor pressure (WVP) trace with corresponding oxygen data traces of one analyzer (VO_2 channel 1) (A) and z-Transformed oxygen data (VO_{2_c}) (B), respiratory quotient (RQ_c) (C) and energy expenditure (EE_c) (D) after adjusting the setup settings of the air conditioner.

Temperature of gas analyzer-unit

According to the construction plan of the gas analyzer-unit the two gas analyzer chains are placed in a case that is heated to a constant temperature of 37°C in order to guarantee a stable environment for the gas analyzers and to avoid discrepant measurement conditions between the two analyzer chains. Maintaining a constant temperature is in particular important for the fuel-cell O₂-analyzer because of its temperature sensitivity [84]. Although the analyzers are equipped with a thermal compensation array to reduce temperature effects, particular attention must be paid to the rate of temperature changes, since the array and the fuel cell electrolyte have different thermal time constants [84]. Therefore, the inner wall of the gas analyzer-unit is coated with polystyrene to isolate the unit. Nonetheless, the temperature recordings of both analyzer units showed rapid fluctuations, which were visible in the raw data traces as well as in the processed datasets. **Figure 7** shows the coincidence of the temperature change with an unphysiologically spike in the measured $\dot{V}O_2$ and the response time corrected RQ.

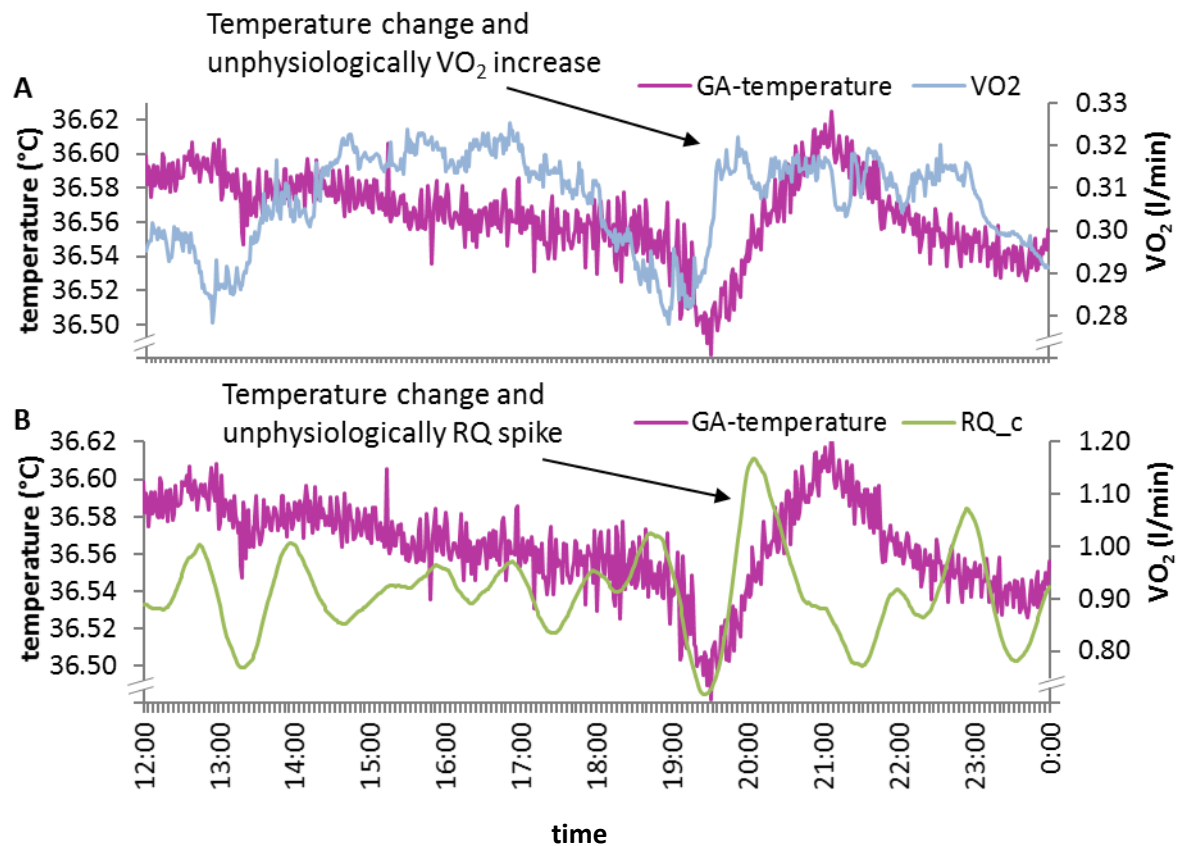


Figure 7 Gas analyzer-unit temperature with fluctuation (GA-temperature) and corresponding oxygen data ($\dot{V}O_2$) (A) and z-Transformed respiratory quotient (RQ_c) (B). The coincidence of the temperature change and an unphysiologically increase in the measured $\dot{V}O_2$ and the calculated RQ_c is shown.

After closer inspection of the gas analyzer-unit environment, the reason for this massive disruption became obvious. The gas analyzer-unit was placed on a table in a small usually closed room in front of the chambers. The air conditioner of the room, where the metabolic chambers are installed is located directly on the opposite side of the gas analyzer-unit room. When the door of the analyzer room was opened and not immediately closed again, the cold airstream of the air conditioner flowed towards the analyzer-unit and caused temperature changes within the gas analyzer-unit. This inappropriate positioning of the air conditioner caused no problems during test runs because the gas analyzer-unit room door was usually closed. However, during the study, crossing of the gas analyzer-unit room was inevitable in order to provide the participants with food, beverages and examination devices through the locks.

After the reason for the gas analyzer temperature fluctuations was found, the study team took special care to keep the door of the gas analyzer-unit room closed. In Addition, the analyzer units were further isolated with polystyrene from the outside of the analyzer units (**Figure 8**). Considering these difficulties, it is advisable to find a thermally shielded place for the analyzer units.



Figure 8 Gas analyzer units covered with polystyrene to avoid temperature changes within the analyzer units

Response time correction: time resolution vs. noise reduction of the data

Because of the reasons mentioned above and trouble with the ventilation of the building, the raw data were sometimes noisy and did not reach perfect quality. Consequently, some data sets were extremely noisy after z-Transformation (see *Acquisition and processing of raw data*). Therefore, to analyze the data, it was necessary to smoothen it not just before but also after z-Transformation. Data smoothing was conducted by a so-called moving average filter using ExpeData software. The moving average filter is a simple and commonly used approach in digital signal processing. As the name implies, the moving average filter operates by averaging a number of points from the input signal to produce each point in the output signal [100]. **Figure 9** shows an exemplary dataset where three different post hoc smoothing windows (600, 1200 and 1800) were tested and examined by visual inspection. As described above, smoothing the data always leads to a loss in data resolution. Thus, it is important to find a sound trade-off between noise reduction and resolution. Depending on the final outcome parameter, a high resolution is not mandatory, for example to calculate daylong energy or macronutrient oxidation values. However, to investigate short-term changes in energy expenditure or macronutrient oxidation due to interventions, the smoothing degree has to be chosen carefully. If the highest of the three tested degrees of smoothing is used (Figure 9D), relatively noisy data can be evaluated without too much loss of resolution. The finally chosen smoothing degree can be integrated into the macro code to ensure a comparable data analyzing procedure.

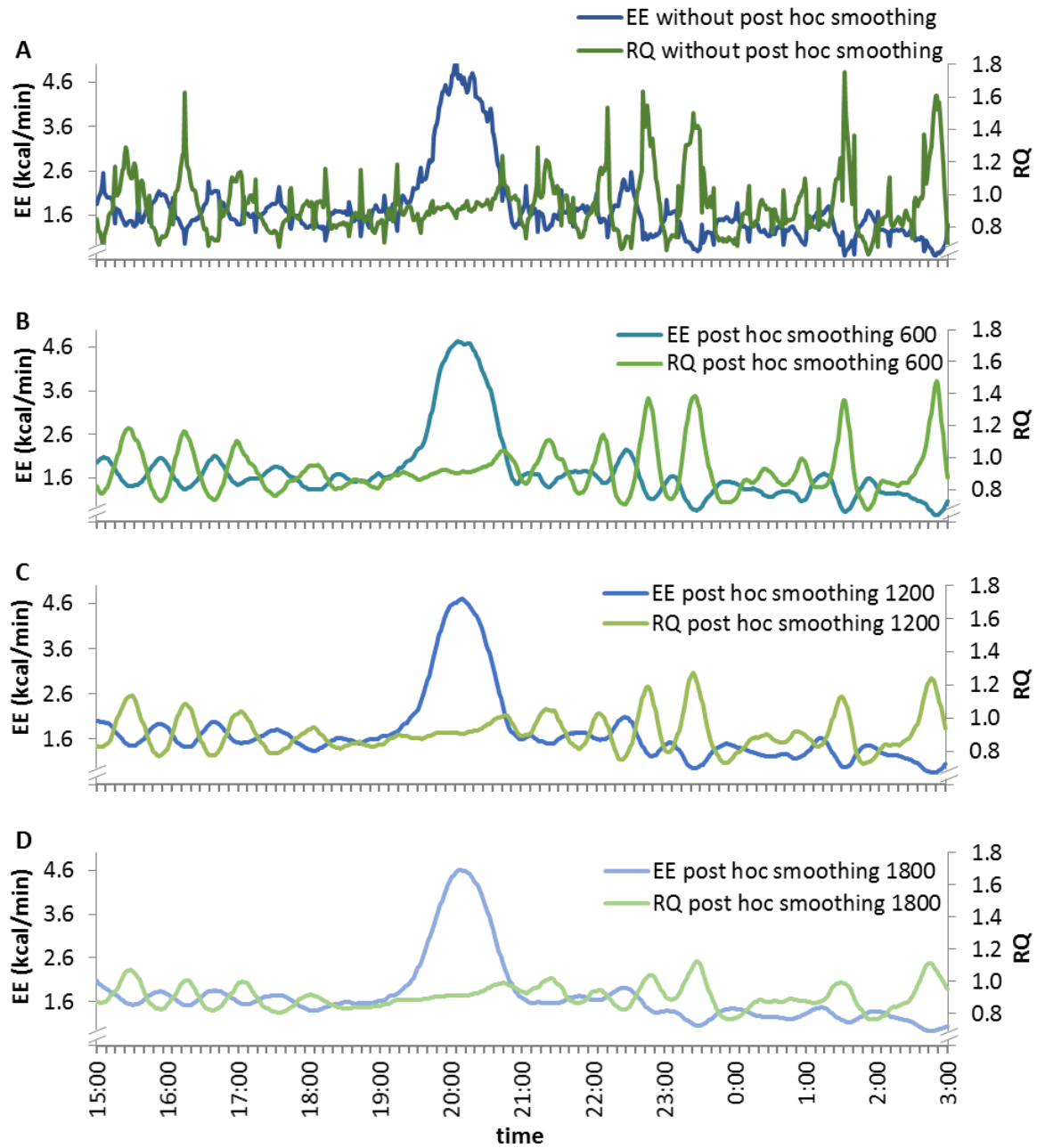


Figure 9 Comparison of response time corrected energy expenditure (EE) and respiratory quotient (RQ) data between three different degrees of post hoc smoothing on an exemplary dataset: Dataset without post hoc smoothing (A), with a smoothing window of 600 (B), 1200 (C) and 1800 (D).

Discussion of methods

The present studies (*Chapter III* and *IV*) in this thesis were highly controlled nutrition and physical activity interventions conducted in a metabolic chamber. Although the methods for assessing the data of the metabolic chambers of these studies have been carefully chosen, there are some limitations. In the following section, the strength and weaknesses of these methods are discussed.

Limitations of metabolic chambers for assessment of rapid changes in energy expenditure and macronutrient oxidation

Accurate assessment of rapid changes in the variables measured with a human metabolic chamber, assumes a profound knowledge and implementation of the system to minimize any noise in the raw data. Given that the signal to measure (about 0.4-0.5 l/min for O₂) is very small compared to the size of the chamber (21,000 l), there is a problem of a low signal to noise, from which all human metabolic chambers suffer [35]. In essence, achieving a high and accurate time resolution depends on the one hand on reducing the noise in the system and on the other hand on the algorithm for correcting the response time. In the early past, several groups of scientists have developed approaches to reduce measurement noise and to improve the response time of the metabolic chambers on this basis [32,35,36,101]. In the present thesis, the z-Transformation method was used, based on a linear combination of recorded gas concentration and its derivative (*Chapter II*). This method allows a real-time metabolic rate calculation (Moon et al., 1995), but the accuracy of the method depends on the derivative terms, which introduce noise when the response time is shortened (higher z-value, see *Acquisition and processing of raw data*) [91]. Therefore, in the present metabolic chambers, a moving average filter was used to reduce the noise in the data by data smoothing. This filter has shown a sufficient overall recovery (90.7 – 107.6%) and mean square error for the time course of O₂ consumption of a known input signal when tested in an *in silico* simulation, nevertheless, the time course of estimates was found to be erratic and under-damped [36]. Being under-damped describes, that the estimates are oscillating with decreasing amplitude until reaching the actual value [102]. This is, in particular, a limitation in regard to estimating RQ values in a

short interval [36] because the accuracy of RQ depends on absolute values since it is a ratio of two variables [39]. As described above, the metabolic chamber data in this thesis suffered from noise in the system. Figure 9 demonstrates that z-Transformation on this data was especially critical for assessing short-term RQ data, which in fact were very erratic. In a recent study, a so-called “wavelet de-noising technique” was tested in combination with a “central difference method” [32]. The wavelet de-noising filter was able to smooth the noise during steady-state after applying the central difference method, while at the same time detailed short-term changes could be retained [32]. This means that unwanted noise is suppressed while important information is retained without smoothing out details [32]. An implementation of the wavelet de-noising filter in the present data processing procedure, might, therefore, be able to dampen the erratic character of the data, resulting in improved time resolution.

Beyond all the published methods that demonstrated their ability to measure short-term dynamics of oxygen consumption or carbon dioxide production rates, only one recent publication demonstrated the reliability for recovery of short-term substrate oxidation data [39]. In this publication, data deconvolution was solved using an “augmented regularization technique”. The results have shown that minute-to-minute measurements of substrate oxidation can be performed with statistically meaningful consistency. Besides all the limitations mentioned above, in the present thesis substrate oxidation data of the study on energy flux (*Chapter IV*) and meal skipping (*Chapter III*) confirmed the results of Gribok et al. [39] and demonstrated that the acute response in substrate oxidation to diet and exercise challenges can be measured in metabolic chambers.

Although there are various algorithms applied to human metabolic chamber data, there is still a paucity of comparison under different conditions [36]. In particular, because of the diversity of the existing methods and metabolic chamber systems, further research has to be done to evaluate and to further improve the measurement of short-term dynamics using metabolic chambers.

Assessment of total energy expenditure and macronutrient oxidation

Total energy expenditure has shown to be measured reproducibly in a metabolic chamber with a coefficient of variation within subjects of 1-5% [6,103–105]. Thus, it is considered one of the most accurate outcome parameters using a metabolic chamber [28]. Using the Hohenheim metabolic chambers within-subject coefficient of variation was determined for three different PALs. This was obtained from repeated 24-h measurements of 11 participants with three different activity protocols for the stay in the metabolic chamber. Within-subject coefficient of variation for a PAL of 1.3, 1.5, and 1.7 was 2.0%, 2.7% and 3.0%, respectively.

Energy expenditure was calculated using the Weir equation [96]. This equation calculates energy expenditure from respiratory gas exchange and is based on the assumption of metabolizing a mixture of carbohydrate, fat, and protein. Since the energy equivalent of oxygen consumed depends on the macronutrients that are being oxidized, the accuracy of this approach is limited when other nutrients than carbohydrate as glucose polymer (starch or glycogen), dietary fat and protein are metabolized as for example glucose monomers, ketone bodies, and alcohol [106]. During both studies of the present thesis, participants received a mixed diet with a constant macronutrient proportion and were not allowed to consume alcoholic beverages. The use of the Weir equation can, therefore, be considered appropriate. However, in the studies of this thesis, the participants mainly received convenience products as well as sweets and thus also glucose monomers. In the case of the ingestion and subsequent oxidation of glucose monomers, inaccuracies in calculations of about 0.5% occur [106].

By contrast, the occurrence of lipogenesis and gluconeogenesis does not disturb the calculations, because the general principle in the calculation is that the energy expenditure is determined by the reactants and the products of combustion [107] and is independent of intermediate metabolic processes [24]. In the case of lipogenesis from carbohydrates, a negative fat oxidation is counterbalanced by an increased oxidation of carbohydrates [108]. The occurrence of gluconeogenesis involves the conversion of lactate, pyruvate and, glycerol to glucose, which is independent of gaseous exchange (cori cycle) and thus is not relevant when there is no loss or accumulation of this intermediates within the body [92]. When alanine, as the major non-carbohydrate substrate for

gluconeogenesis is converted into glucose, this process can also be balanced [24]. If it is assumed, that the oxidation of fat accounts for the energetic cost of glucose formed from alanine, and the produced glucose is also oxidized (calculated rate of glucose oxidation is the net balance of glucose oxidation and glucose synthesis) [109], gluconeogenesis only leads to a minor error in terms of energy expenditure calculations [92]. In both studies of this thesis, gluconeogenesis might have occurred, since a prolonged fasting period (*Chapter III*) and a negative energy balance in combination with low-intensity physical activity (*Chapter IV*) was included in the study design. However, it was shown, that prolonged exercise (4 h) in the fasted state that resulted in an 8% contribution of alanine on glucose production only accounted for no more than 1% of energy expenditure [110].

Furthermore, it has been shown that the incorporation of a protein oxidation correction factor has only a negligible impact on the accuracy of the calculation of energy expenditure, in particular when the percentage of protein consumption is within a usual range of 8-20% [96,106]. Nonetheless, this correction was included in the calculations in both studies of this thesis.

Although the energy equivalent of oxygen consumed by the oxidation of protein and fat have been revised [108] the Weir equation is still accurate to within 1 % [106]. Alternative calculations of metabolic rate by determining the proportion of carbohydrate and fat from the npRQ were found to be more inaccurate [106].

Considering the limitations of indirect calorimetry to assess substrate oxidation, it must be emphasized again that the value for the volumes of oxygen consumption for the oxidation of 1g of carbohydrate or fat depends on the type of substrate [106]. Thus, the amount of oxygen consumed by the oxidation of glucose (monomer, 0.746 l O₂) differs from that of glycogen (0.829 l O₂), which is the carbohydrate energy source in the post-absorptive state [24]. In this thesis, the value for glucose was used to calculate carbohydrate oxidation. In the case of fat and protein, the values for the amount of oxygen consumed by different types of substrates are very similar [24]. In addition, the occurrence of gluconeogenesis from alanine should be considered, since the contribution of alanine deamination to nitrogen excretion leads to an overestimation of protein oxidation. Glucose oxidation is underestimated because the calculated value represents

glucose oxidation minus glucose synthesis, and fat oxidation is also underestimated to 0.09 times the rate of gluconeogenesis [109].

Assessment of sleeping energy expenditure

Sleeping energy expenditure is a major outcome parameter in metabolic chamber studies because metabolic chambers are the unique method to measure SEE in almost free-living conditions without facemasks or hoods [111]. The variability in SEE, measured by metabolic chambers, is about 2% [103,104,112]. Because in contrast to REE, SEE is less affected by activity within the body, it has a high degree of reproducibility [28] and, therefore, represents a relatively unaffected outcome parameter free from confounding factors. Nevertheless, sleep stages, seasonal cycles, temperature, DIT, daily physical activity, and body movement during the night have been shown to modulate SEE [6,104,113–115]. Dependent on the study hypothesis these factors should be controlled.

For the assessment of SEE different methods were used in the literature. In many cases SEE can be calculated (i) as the energy expenditure during the time period when activity is the lowest [116], (ii) as the energy expenditure value over a 3-h interval with the lowest residual energy expenditure, which was calculated as the residual of the individual relationship between EE and physical activity [111] or (iii) as the lowest energy expenditure value over a period of 3 h of continuous sleep [97]. As shown in the study of Schoffelen et al. [111] the latter measurement method resulted in a coefficient of variation of only $1.8 \pm 1.4\%$ and, therefore, can be considered as valid. Although there was no significant difference in SEE between the three methods, the authors recommended using the method (ii), when physical activity measurements were performed.

In the study on energy flux in the present thesis, SEE was calculated using method (iii). This method was chosen because its calculation does not depend on the use of a continuous activity monitoring device. In order to measure the impact of the daytime intervention on SEE, the night following the intervention day in the metabolic chamber was used to examine SEE. In contrast, Schoffelen et al. emphasized to use nights following free-living conditions because it was observed that overnight metabolic rate was increased and SEE tended to be higher on the second night compared to the first night of

a 36 h stay in the metabolic chamber [111]. This increase was related to increased physical activity during the night period and was attributed to behavioral adaptation [111]. Evaluation of SEE in the energy flux study was, however, always performed on the second night when participants were already accustomed to the metabolic chamber.

In addition, it should be considered that an increase in SEE could also reflect an ongoing DIT that continues into the night [117] (see *Assessment of DIT*). This was supposed by the fact that energy expenditure after a meal did not return to the pre-prandial values within 4 h. Since energy content of the dinner during the energy flux study varied between intervention days (*Chapter IV*) and the magnitude of the increase in DIT is influenced by the energy intake (for review see [118]), this can be considered as a possible explanation for the observed differences in SEE.

Assessment of DIT

Depending on the method used to determine DIT, there is a poor reliability of DIT with an intra-individual coefficient of variation of 6%-48% [6,119–121]. The lower variability was reached using a metabolic cart, where participants were in a supine position and only short time periods of three h were assessed [120,121]. Measuring DIT using metabolic chambers has the advantage of more physiological conditions [98,119] and to cover with a greater probability the whole DIT period, which was shown to last far beyond four hours [117,122–124] and even up to eight hours after the ingestion of food [125].

In general, DIT is defined as the increase in energy expenditure in response to food intake [126] and can be estimated as the difference between energy expenditure without physical activity and basal metabolic rate [28]. There are two different approaches to calculate DIT. The approach of Tataranni et al. is often considered the gold standard, but it requires two separate measurements since the difference in TEE between the fed and the fasted state is computed [119]. The second approach is a simplified method that was proposed by Schutz et al. and estimates DIT as the difference between the energy expenditure at zero activity (EE_0 = y-intercept of the linear regression between energy expenditure and physical activity) and basal metabolic rate [99]. The method was modified and used commonly by several working groups [6,98,119,127]. Modifications were performed with regard to the measuring period and the use of SEE instead of basal

metabolic rate. Using SEE instead of basal metabolic rate results in a more reproducible baseline [6]. Furthermore, using a calculation period of DIT of 24 h, compared to 15 h during daytime was found to better correlate with the method of Tataranni et al. (DIT = difference in TEE between fed and fasted state) [119]. This could be attributed to the fact that, in particular after ingestion of a dinner, DIT may last much longer and is therefore not covered by a 15 h daytime period [98].

For the present thesis, a modified version of Schutz's method that was applied by Westerterp et al. was used [98]. This method plotted energy expenditure against radar output, both averaged over 30 min periods. In the present thesis DIT was calculated on the basis of physical activity measured by step count via a triaxial accelerometer (ActivPAL, Paltechnologies Ltd., Glasgow, UK), instead of radar output. Using this accelerometer, 1 h averages of step count were used to calculate the regression line between energy expenditure and physical activity. This is a limitation because any physical activity that was not reflected by step count was not considered and distorts the DIT values. Consistent with the radar output the accelerometers did not provide information on the intensity of activity. Furthermore, instead of a 24 h period, only 23 h DIT was calculated and then extrapolated over 24 h. However, this should not have any significant effect.

A further problem calculating DIT in the study on energy flux (*Chapter IV*) was that during medium and high EF low-intensity physical activity (walking on a treadmill with 4 km/h) was performed at three distinct time periods and in-between participants were required to remain sedentary. This led to single extreme points influencing the slope of the relationship between energy expenditure and step count and consequently to an erroneous determination of the y-intercept. **Figure 10** shows an example of the calculation of DIT for one participant from the energy flux study (*Chapter IV*). Figure 10A demonstrates the calculation of DIT on an intervention day when the participant was sedentary. Figure 10B and C show the data of the medium as well as the high EF day and illustrate the above-described problem. These two days differed in walking time and energy intake, but the calculated DIT was almost the same. It can be hypothesized that the extreme data points lead to this unphysiologically result. Therefore, in order to appropriately calculate DIT using metabolic chamber measurements, a normal

distribution and relatively large range of physical activity-related energy expenditure is needed to eliminate the erroneous calculation of a regression line that is influenced by a few extreme points [98]. In the present thesis, this problem was enhanced by using hourly averages of step count and energy expenditure resulting in fewer data points compared to using 15 min [119] to 30 min averages of physical activity [98].

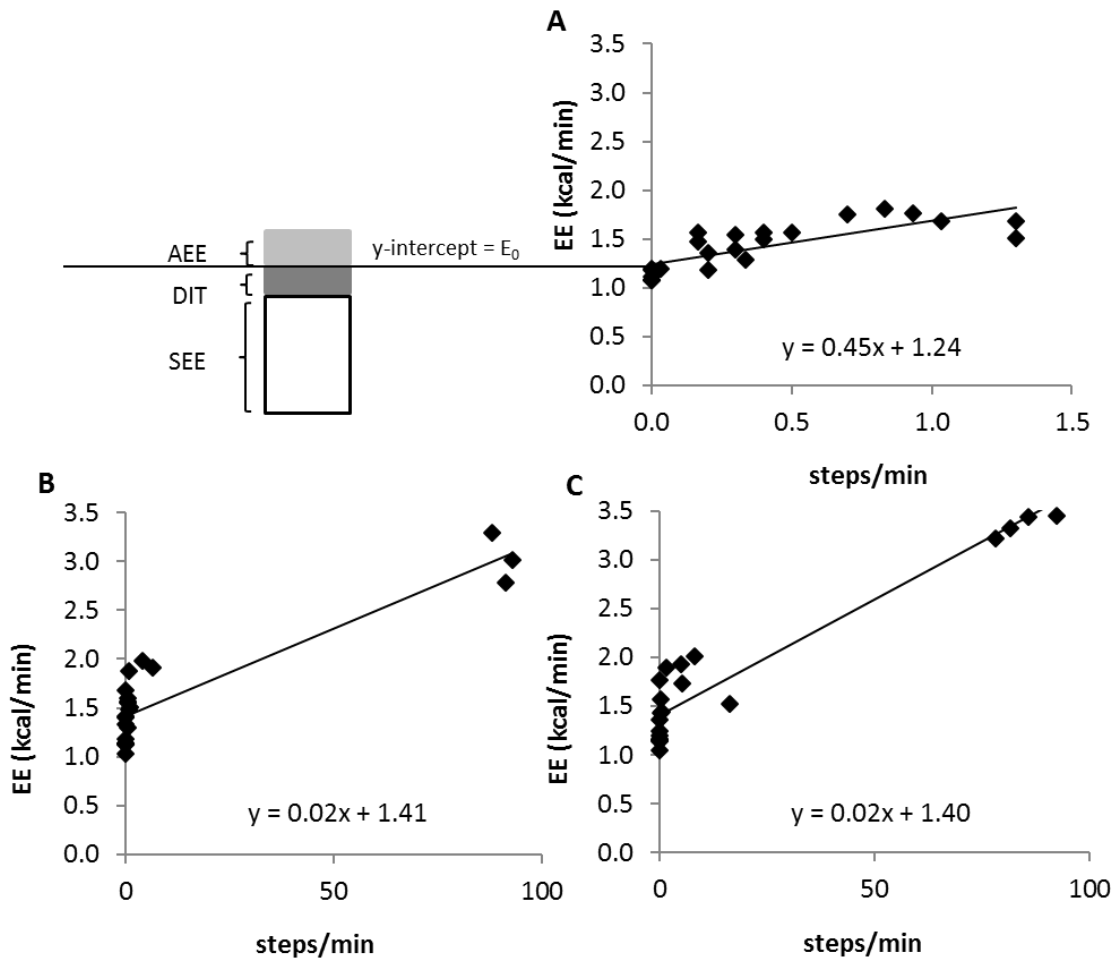


Figure 10 Calculation of diet-induced thermogenesis (DIT) for one participant on a day with sedentary behavior (A), with an activity protocol of 3x55 min walking (B) and with 3x110 min walking with 4km/h (C). Between walking periods, the participants were required to remain sedentary, which leads to a few extreme points, that influenced the determination of the y-intercept in (B) and (C). The calculation was performed according to Westerterp et al. 1999.

Calculation of DIT is crucial because quantification of each component of energy expenditure is still challenging in particular in regard of distinguishing DIT from non-exercise activity energy expenditure. Non-exercise activity energy expenditure includes the so-called fidgeting [128] or prolonged increase in energy expenditure due to physical activity in case of exercise interventions [117]. In a more recent publication, a new method for calculating DIT is proposed, that focusses on these points [117]. DIT was estimated as the postprandial increase in energy expenditure free from non-exercise energy expenditure, which was derived from its linear regression on integrated physical activity (measured at 1-min intervals). Comparing this method with Schutz's original method [99] and the modified Schutz's method of Westerterp et al. [98] calculated over 4 h, resulted in the closest match with the method of Tartaranni et al. [119]. Therefore, the use of this method can be recommended for future studies on DIT.

Nevertheless, the method used for calculating DIT in the present thesis was valid because it did not result in negative values for DIT that were observed in other studies in particular when DIT was calculated as EE_0 minus basal metabolic rate, instead of SEE [6,117]. Absolute and relative values of DIT (DIT as a percentage of energy intake) on low EF intervention days (see *Chapter IV*) were comparable to values in the literature [117,119] with a range of 11%-23% of energy intake between individuals. Because SEE is known to be 5% smaller than basal metabolic rate [94], it could be considered to adjust the calculated DIT values for this difference in order to obtain values that are more physiological and to obtain a better comparison to the values of studies that used basal metabolic rate to calculate DIT [4].

Conclusion

Metabolic chambers are the current gold standard to continuously measure energy expenditure and macronutrient oxidation. The unique opportunity to assess acute energy and macronutrient balances will contribute to the research on the development of obesity. As major methodological improvements have been made, short-term kinetics in energy expenditure and macronutrient oxidation can be measured. Nevertheless, data processing in terms of response time correction is still challenging. This thesis showed, that in order to obtain sound measurements, thorough considerations must be made in terms of the metabolic chamber environment, the additional devices (e.g. air conditioner) used as well as the study protocol.

Chapter III

Impact of breakfast skipping compared with dinner skipping on regulation of energy balance and metabolic risk

The study of this chapter was previously published [129]. For this thesis, the metabolic chamber data of the study were reanalyzed for 13 participants using the data processing procedure described in chapter II of this thesis. In the publication, z-Transformation was not performed but is now included in the data analyzes.

Subjects and Methods

Seventeen healthy adults (9 women, 8 men) were recruited by notice board postings at the Universities of Hohenheim and Stuttgart between October 2015 and April 2016. Exclusion criteria were food allergies or intolerances, alternative nutrition habits, smoking, chronic diseases or regular use of medications. Thirteen participants were regular breakfast eaters and four were occasional breakfast skippers. The terms "breakfast eaters" and "skippers" were not further defined to participants or by investigators. The study protocol was approved by the ethics committee of the Medical Council of Baden-Württemberg, Germany. The trial was registered at clinicaltrials.gov as NCT02635139. All of the participants provided written informed consent before participation.

Study protocol

A randomized crossover nutrition intervention was conducted at the Institute of Nutritional Medicine at the University of Hohenheim. An outline of the study protocol is given in **Figure 1**. A 3-day run-in period with a controlled diet preceded the intervention phase to adapt macronutrient oxidation to macronutrient intake [130]. On the intervention days participants consumed isocaloric diets (55% carbohydrate, 30% fat, 15% protein) with three 24-h conditions: i) a conventional three-meal-structure day (control), ii) a breakfast skipping day (BSD) and iii) a dinner skipping day (DSD). The BSD and DSD were randomly assigned, and the first skipping day was followed by a washout day to again obtain a constant fasting period of 18 h before the next intervention day. Thus, the sequence of the intervention days was either BSD-washout-control-DSD or DSD-washout-control-BSD. Participants were randomly assigned by using block randomization to begin with BSD or DSD intervention in a 1:1 allocation ratio that was based on a computer-generated list of random numbers. The study team enrolled and assigned the participants to the interventions. During the entire metabolic chamber period participants followed a constant daily routine: wake up at 0600, meals at 0700, 1300 and 1900 and bedtime at 2200. On the day before the first intervention day participants were admitted to the institute at 1830 to install a continuous glucose monitoring sensor. Participants spent the

night before each intervention day in the metabolic chamber and left the morning after the intervention day. On the washout day participants were allowed to go home for 12 h. During the intervention days, blood samples were collected frequently from 0700 to 2100 to measure free fatty acids (FFA), ghrelin and cortisol concentrations (ghrelin and cortisol were determined in a subsample of 8 participants and ghrelin was measured at the skipping days only). The first blood sample at 0700 was taken in a fasting state. After lunch on the skipping days, blood was sampled every 30-minutes for 2 h for the determination of glucose and insulin concentrations.

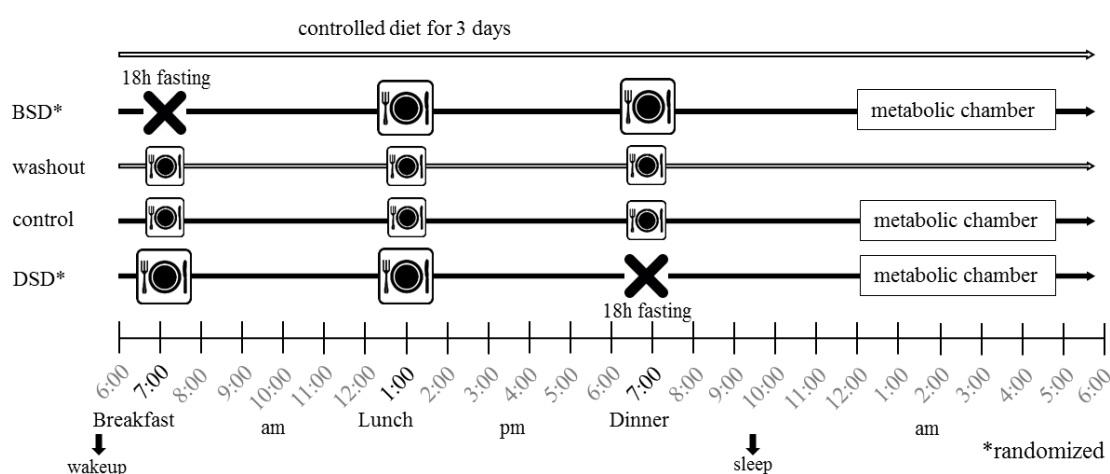


Figure 1 Schematic overview of the study protocol. *Randomly assigned. BSD, breakfast skipping day; DSD, dinner skipping day.

Control of energy intake and physical activity

During the whole study period participants all foods were provided from the Institute of Nutritional Medicine's metabolic kitchen. Participants were instructed to only consume the provided food, water, and unsweetened tea and to refrain from vigorous physical activity. During the first 2 d of the 3-day run-in period, participants ate ad libitum and leftovers were back weighed to calculate dietary intake. On the other study days, all provided food was consumed and participants were required to remain sedentary. Macronutrient composition was kept constant throughout the entire study period and for each meal. On intervention days, participants received the same food items each day. Individual diet composition was calculated by using Prodi6 software (Wissenschaftliche

Verlagsgesellschaft, Stuttgart, Germany). Energy intake was based on individual energy requirements to obtain energy balance. Skipped meals were therefore compensated for by equally increased energy content of the other 2 meals on this day (equal energy content of both remaining meals). On the control day, each of the 3 meals had the same energy content. Individual energy requirement was calculated on the basis of resting metabolic rate measured by open-circuit indirect calorimetry in the morning after an overnight fast (ventilated hood system, Quark RMR, COSMED, Rome, Italy) before to the study period and multiplied by a PAL of 1.35 as estimated for the days in the respiratory chamber. Physical activity was continuously measured by using a triaxial activity monitor (ActivPAL, Paltechnologies Ltd., Glasgow, UK). The time spent sitting/lying, standing, stepping and the step numbers were analyzed. The ActivPAL was worn at the mid-line of the thigh, one-third of the way between hip and knee and fixed with a waterproof tape according to the recommendation of the manufacturer.

Body-composition analysis

Examination took place before the 3-day run-in period after an overnight fast. Height was measured with a stadiometer (Seca 274, Seca GmbH & Co. KG, Hamburg, Germany). Body weight was measured on a calibrated impedance scale (Seca mBCA 515, Seca GmbH & Co. KG, Hamburg, Germany). Fat mass was assessed by using air displacement plethysmography via the BodPod Body Composition System (COSMED, Rome, Italy). Fat mass index (FMI) was calculated as fat mass divided by the square of height (kg/m^2).

Twenty-four-hour energy expenditure and substrate oxidation

The metabolic chambers and the assessment of TEE and 24h-macronutrient oxidation were described in *Chapter II*. The FR of the metabolic chambers was determined as 80 l/min. Energy and macronutrient balance was calculated by subtracting energy expenditure and macronutrient oxidation from the respective intake. On 2 days, technical problems with the power supply occurred. The metabolic chamber data for these 2 participants were excluded for all days because of the crossover study design with

intraindividual comparisons. Data from 2 other participants were excluded from the final analysis because of problems in the data acquisition and processing procedure as described in *Chapter II* of this thesis.

FFAs were measured photometrically and total area under the curve (tAUC) was calculated for 14 h (0700-2100).

Assessment of glucose metabolism and hormonal measurements

Interstitial glucose concentrations were measured continuously using the Dexcom G4 glucose-monitoring device (Dexcom G4 Platinum, Nintamed GmbH & Co KG, Mainz, Germany) during the entire room calorimeter phase. The sensor was applied to the back of the upper arm to measure interstitial glucose concentrations in the subcutaneous tissue. Sensor readings were reported every 5 min. The device was calibrated twice a day using capillary blood samples. AUC was calculated as tAUC for the entire intervention days (0600-0600) by using trapezoidal rule [131]. Glucose variability was assessed by the mean amplitude of glycemic excursions (MAGE) [132] by using a published macro [133].

Glucose was measured by using hexokinase method and serum insulin was determined by electrochemiluminescence. Incremental AUC (iAUC) was calculated using trapezoidal rule [131] for 2 h postprandial after lunch on BSD and DSD. Homeostasis model assessment-insulin resistance (HOMA-IR) [134] and postprandial homeostasis model assessment index (HOMApp) after lunch [135] on BSD and DSD was determined. Postprandial iAUCs and HOMApp after lunch were only assessed on BSD and DSD because of the smaller energy content of lunch on the control day. Twenty-four-hour insulin secretion was obtained by 24-h urinary C-peptide excretion by using luminescence immunoassay method.

Cortisol secretion was determined by luminescence immunoassay, and tAUC was calculated for 22 h (0700-0500). Ghrelin was examined by radioimmunoassay, and tAUC was calculated for 14 h (0700-2100).

SNS activity and autonomic function

Heart rate variability (HRV) was assessed in a continuous electrocardiographic recording (ECG) by using an autonomic nervous system recorder (ANS-Recorder Flex BT; Neurocor Ltd. & Co. KG, Trier, Germany). Measurements were conducted in a sitting position for 5 minutes every 2 h throughout the intervention days and every 30 min after scheduled meal time for 2 h, even when the meal was skipped. ECG recordings were made under a nonstressful situation that we defined as quietly resting in an armchair in the metabolic chamber with dimmed lighting. The ECG signal was inspected for artifacts and analyzed by using corresponding NeurocorV R software (ANS-Explorer V3.5.11, Neurocor Ltd. & Co. KG, Trier, Germany). Time-domain parameters included the SD of normal-to-normal intervals (SDNN; a global measure of overall HRV) and the root-mean-square differences in successive normal-to-normal intervals (RMSSD; a measure of parasympathetic activation). As a marker of sympathovagal balance, the ratio of low frequency (LF; 0.04-0.15 Hz) to high frequency (HF; 0.15-0.4 Hz; LF:HF) was analyzed.

Adrenaline and norepinephrine excretion in 24-h urine were measured using liquid chromatography-mass spectrometry.

Statistical analyses

Together with total energy expenditure, fat oxidation was the primary outcome parameter of the study. It was therefore used for power analysis. According to the main hypothesis, fat oxidation was compared between BSD or DSD and control day. Power analysis was conducted using G-Power 3.1.9.2 Software (written by Faul F., University of Kiel, Germany) and a 2-sided *t* test for difference between 2 dependent means and an α level of 0.05. Means \pm SDs for fat oxidation (61.9 ± 4.6 g/d) were based on the data of Munsters et al. 2012 (6). In order to show a 6% difference in fat oxidation with a power of 80% a total sample size of $n = 13$ is required. Data are reported as means \pm SDs unless otherwise specified. Normal distribution was checked by Kolmogorov–Smirnov test. Repeated-measures ANOVA was used to examine differences in the variables of energy- and macronutrient balance, glucose metabolism, HRV data, and catecholamine and cortisol concentrations between the 3 intervention days. Significant effects were followed

with pairwise comparisons and Bonferroni post hoc tests. Differences between the skipping days in insulin, glucose, and ghrelin concentrations were analyzed by paired *t* test and Wilcoxon's test was used if data were not distributed normally. Sex differences in baseline characteristics were analyzed using independent-samples *t* test. Differences between regular breakfast eaters and occasional breakfast skippers as well as between participants with a low and high fat mass index were tested by Mann-Whitney *U* Test. Correlations between npRQ and C-peptide, FFA tAUC, RMSSD, adrenaline, or norepinephrine as well as correlations between TEE and adrenaline or norepinephrine were tested by Spearman's ρ and included the data of all intervention days. All analyses were conducted by using SPSS statistical software (version 23; SPSS, Inc., Chicago, IL, USA). Significance was set at $P < 0.05$.

Results

Baseline characteristics of the study population are shown in **Table 1**. Eight women and 9 men aged 20–31 y participated in this study. BMI (in kg/m²) and percentage of body fat mass ranged between 18.3–35.0 and 7.4% and 33.9 %, respectively. According to WHO criteria, 3 participants were overweight, 2 were obese and 1 was underweight.

Dividing man and women into 2 groups according to their mean FMI showed that there were no differences in meal skipping-induced changes between the 2 groups in postprandial glucose iAUC, insulin iAUC and HOMApp after lunch; 24-h glycemia; C-peptide excretion; TEE; or 24-h fat oxidation. However, our study was not powered to detect differences between subjects with lower and higher FMI. Therefore, these analyses should be interpreted with caution.

Table 1
Baseline characteristics of the study population¹

	Women n=9	Men n=8	Total n=17	<i>p</i> ²
Age, y	23.7 ± 2.5	25.6 ± 3.9	24.6 ± 3.3	NS
Height, m	1.64 ± 0.06	1.83 ± 0.08	1.73 ± 0.11	<0.001
Body weight, kg	57.7 ± 7.9	88.0 ± 17.3	71.9 ± 20.1	<0.001
BMI, kg/m ²	21.2 ± 1.9	26.6 ± 5.2	23.7 ± 4.6	0.022
FMI, kg/m ²	5.7 ± 1.6	6.2 ± 3.6	6.0 ± 2.6	NS

¹Values are means ± SDs. FMI, fat mass index.

²*p*-value for sex differences tested by using independent-samples *t* test.

Energy and macronutrient balances

Energy intake (kcal) was similar by design for the 3 intervention days (**Table 2**). Total energy expenditure (kcal/d) was higher on the DSD than on the control day. There was no difference in TEE between the BSD and the control day. **Figure 2** shows the daylong profiles of TEE. Energy balance (kcal/d) on the control day was slightly more positive than on the skipping days. However, physical activity did not differ between skipping days and the control day (number of steps (steps/h) – BSD: 655 ± 247 ; DSD: 710 ± 238 ; control: 644 ± 207 ; time spent sitting/lying (h/d) – BSD: 22.3 ± 0.7 , DSD: 22.1 ± 1.1 , control: 22.3 ± 0.8 ; time spent standing (h/d) – BSD: 1.5 ± 0.7 , DSD: 1.7 ± 1.0 , control: 1.6 ± 0.8 ; time spent stepping (h/d) – BSD: 0.2 ± 0.1 , DSD: 0.2 ± 0.1 , control: 0.2 ± 0.1 ; all $n = 15$, $p > 0.05$).

No difference in fasting-npRQ was observed between the 3 intervention days (BSD: 0.82 ± 0.06 ; DSD: 0.80 ± 0.08 ; control: 0.82 ± 0.05 ; $p > 0.05$). Components of macronutrient balance are presented in Table 2. Macronutrient intake was similar between the 3 intervention days by design. When compared with the control day, 24-h fat oxidation was higher and 24-h carbohydrate oxidation was lower on BSD, whereas both variables did not differ from control on DSD. FFA tAUC was higher on BSD and DSD than on the control day. No association was observed between FFA tAUC and 24-h npRQ. Figure 2 shows the profiles of 24-h fat and carbohydrate oxidation. Even after lunch, postprandial fat oxidation on the BSD (12.70 ± 4.82 g/2 h or 0.11 g/min) was higher than on the control day (10.93 ± 4.71 g/2 h or 0.09 g/min; $p < 0.003$) and tends to be higher than on the DSD (11.43 ± 5.42 g/2 h or 0.10 g/min; $p = 0.079$). Fat balance was more negative and carbohydrate balance more positive on BSD compared with the control day (Table 2). On DSD, fat and carbohydrate balances did not significantly differ from the control day. Protein oxidation and balance were both similar between all intervention days.

Table 2

Comparison of components of energy and macronutrient balance and SNS activity between BSD, DSD, and the 3-meal control¹

	Control	BSD	DSD
Energy balance², kcal/d			
Energy intake	2391 ± 427	2356 ± 433	2352 ± 436
TEE	2329 ± 446	2347 ± 466	2398 ± 472*
Energy balance	62 ± 147	9 ± 130*	-46 ± 121***
Macronutrient intake², g/d			
Fat	78 ± 15	77 ± 15	77 ± 15
Carbohydrate	321 ± 59	316 ± 60	316 ± 60
Protein	88 ± 16	87 ± 17	87 ± 17
Macronutrient oxidation			
24-h npRQ ²	0.85 ± 0.05	0.84 ± 0.05**	0.85 ± 0.05
24-h Protein oxidation ² , g/d	83 ± 18	89 ± 17	87 ± 20
24-h Carbohydrate oxidation ² , g/d	259 ± 55	224 ± 54**	250 ± 51
24-h Fat oxidation ² , g/d	104 ± 48	117 ± 50**	113 ± 53
tAUC FFAs mg/dl x 14h	79 ± 21	163 ± 32***	136 ± 36***
Macronutrient balance², g/d			
Fat balance	-25 ± 36	-40 ± 38***	-36 ± 41
Carbohydrate balance	62 ± 76	92 ± 84*	67 ± 82
Protein balance	5 ± 23	-2 ± 14	1 ± 22
SNS activity			
Adrenaline, µg/d	11 ± 3	10 ± 3	10 ± 3
Norepinephrine µg/d	37 ± 13	44 ± 12	44 ± 14
SDNN, ms	55 ± 15	59 ± 18	53 ± 14†
RMSSD, ms	38 ± 16	41 ± 19	36 ± 14
LF:HF, ms	2.8 ± 3.1	2.7 ± 2.5	2.4 ± 2.1

¹Values are means ± SDs; n = 17 unless otherwise indicated. Repeated-measures ANOVA with Bonferroni adjustments was used; BSD or DSD compared with control *p < 0.05, **p < 0.01, ***p < 0.001 and DSD compared with BSD †p < 0.05. BSD, breakfast-skipping day; DSD, dinner-skipping day; FFA, free fatty acids; HF, high-frequency domain; LF, low-frequency domain; npRQ, nonprotein respiratory quotient; RMSSD, root-mean-square successive difference; SDNN, standard deviation of all normal-to-normal intervals; tAUC, total AUC; TEE, total energy expenditure.

²n = 13.

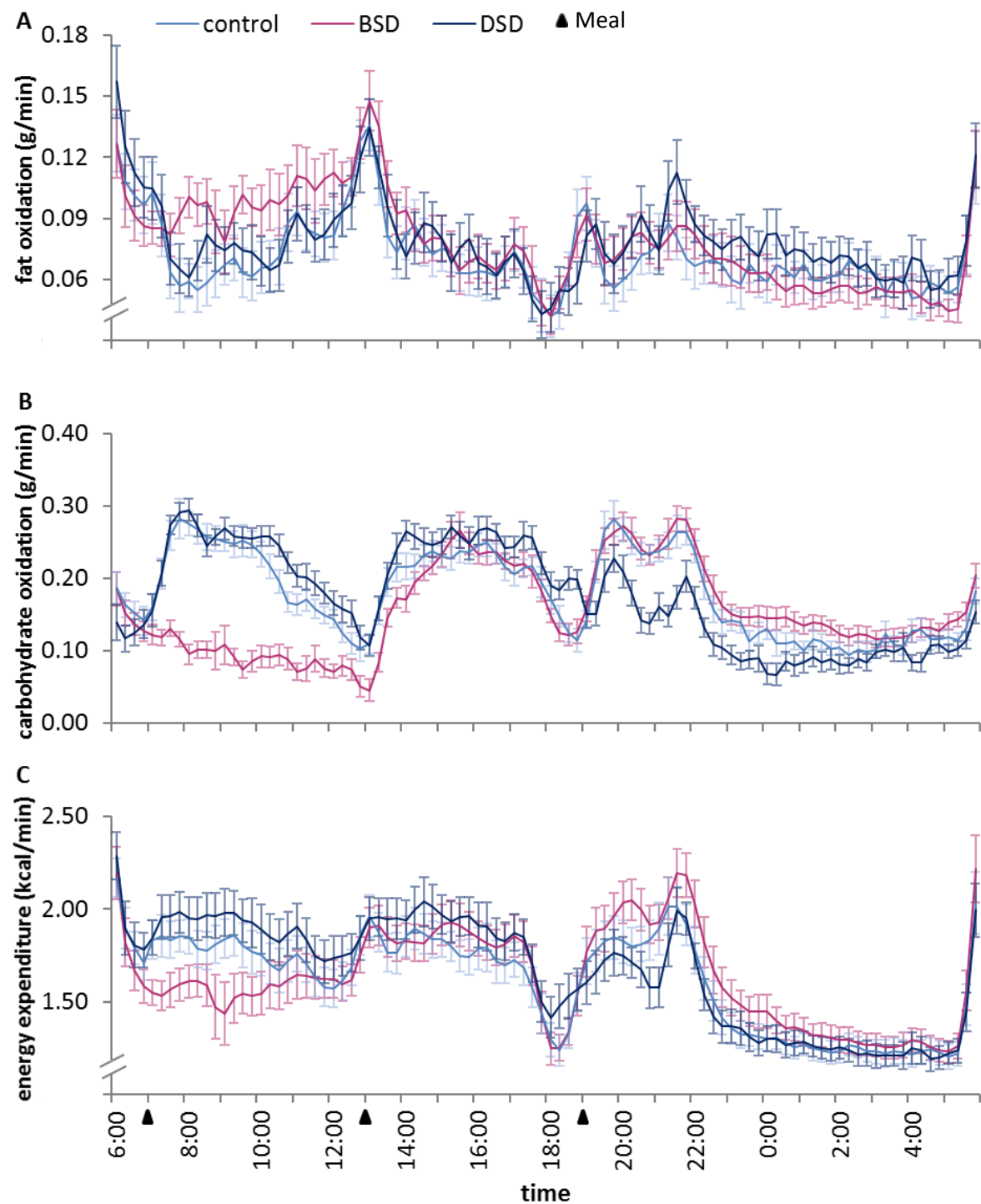


Figure 2 Twenty-four-hour fat oxidation (A), carbohydrate oxidation (B) and energy expenditure (C) for control, BSD and DSD ($n = 13$). Mean values of 15 min were plotted and SEs are shown only at every 30 min for clarity. Differences in the corresponding 24-h cumulative oxidations and total energy expenditure are reported in Table 2. BSD, breakfast skipping; DSD, dinner skipping day.

Impact of meal skipping on autonomic nervous system activity

Although 24-h adrenaline and norepinephrine excretion did not differ between the intervention days, significant differences in diurnal autonomic nervous system activity were observed by heart rate monitoring with a higher RMSSD in the morning on BSD compared to DSD (data not shown). Overall HRV was higher on the BSD than on DSD (SDNN; $p < 0.05$), but not compared with the control day (Table 2). Mean parasympathetic tone (RMSSD) and mean sympathovagal balance (LF:HF) did not differ between the intervention days (both $p > 0.05$).

Twenty-four-hour npRQ showed a positive correlation with parasympathetic tone ($r = 0.50$; $p = 0.001$) and an inverse association with norepinephrine excretion ($r = -0.49$; $p = 0.002$, **Figure 3A**). A higher norepinephrine excretion also correlated with a higher TEE ($r = 0.44$; $p = 0.005$) (**Figure 3B**).

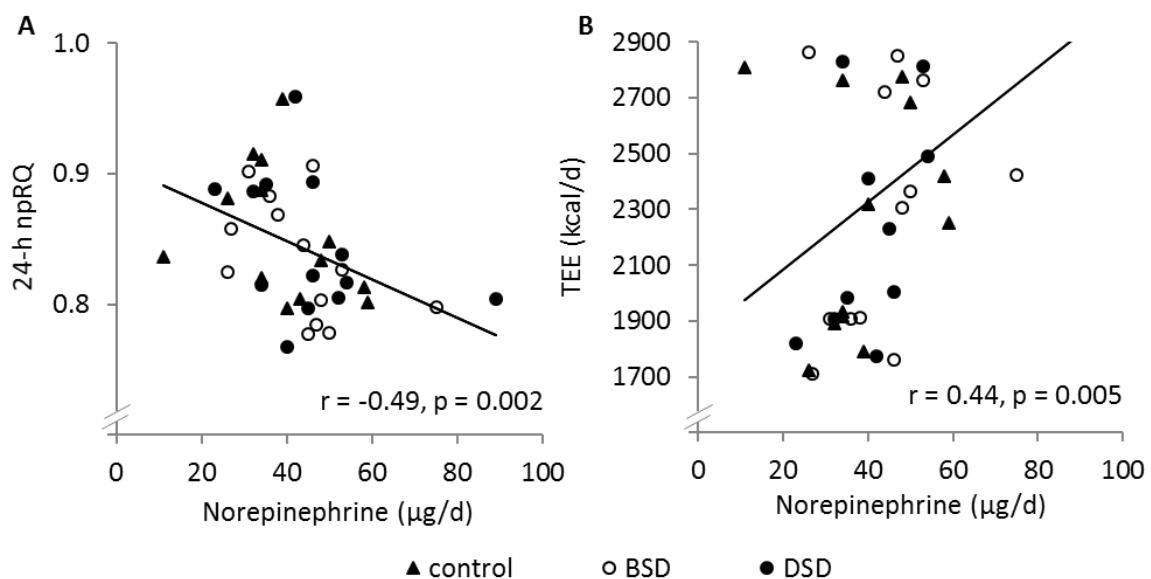


Figure 3 Association between norepinephrine excretion and 24-h npRQ (A) or TEE (B). Correlations include the data of all intervention days ($n = 13$). Spearman's ρ correlation coefficient. BSD, breakfast-skipping day; DSD, dinner-skipping day; 24-h npRQ, 24-hour nonprotein respiratory quotient; TEE, total energy expenditure;

Impact of meal skipping on appetite regulation

Ghrelin concentrations were higher in the morning on BSD and in the evening on DSD day, equalizing one another to a similar ghrelin tAUC between the BSD and DSD (Figure 4).

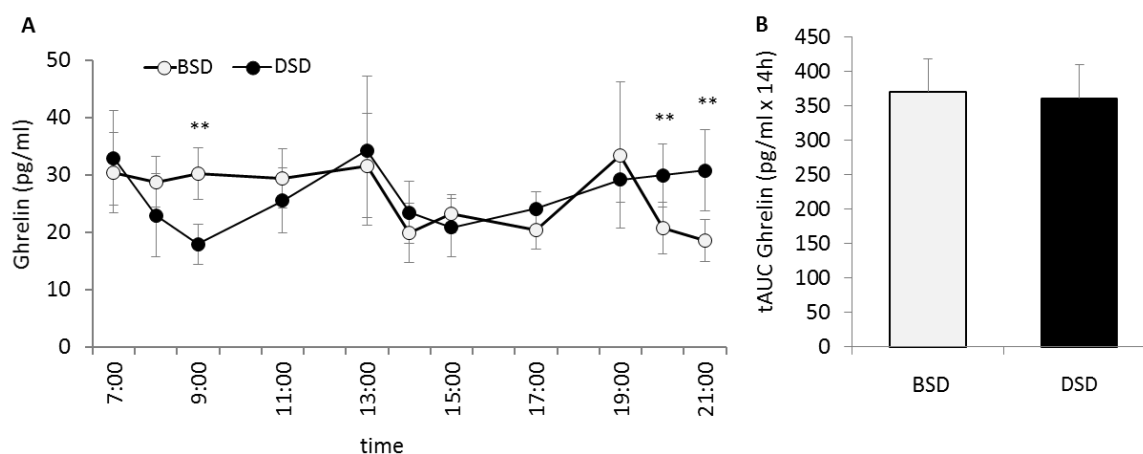


Figure 4 Profile of ghrelin concentrations (A) and comparison of tAUCs (B) between BSD and DSD ($n = 8$). Values are means \pm SDs. ** $p < 0.01$ (paired t test). BSD, breakfast skipping day; DSD, dinner skipping day; tAUC, total AUC.

Impact of meal skipping on 24-h and postprandial glucose metabolism and cortisol concentration

Variables of fasting, 24-h, and postprandial glucose metabolism are shown in **Table 3**. Fasting insulin sensitivity (HOMA-IR), 24-h glycemia (tAUC by continuous glucose monitoring data) and glucose variability (MAGE) as well as 24-h insulin secretion (24-h C-peptide excretion) were all similar between the intervention days. However, when compared with the DSD, BSD resulted in higher postprandial iAUCs of glucose and insulin as well as a higher HOMApp after lunch. No correlation was observed between C-peptide and 24-npRQ. Cortisol tAUC (control: 174 ± 57 $\mu\text{g/dl} \times 22\text{h}$, BS: 181 ± 45 $\mu\text{g/dl} \times 22\text{h}$, DS: 156 ± 40 $\mu\text{g/dl} \times 22\text{h}$; $p > 0.05$) and 24-h cortisol profile did not differ between intervention days.

Table 3

Comparison of the fasting, 24-h, and postprandial glucose metabolism variables between the skipping days and 3-meal-control¹

	Control	BSD	DSD
HOMA-IR ²	1.96 ±0.82	2.07 ±0.91	1.96 ±1.05
24h-Glycemia _{tAUC} ² , mg/dl x 24h	2360 ±111	2425 ±131	2374 ±165
MAGE ²	3.90 ±1.32	3.65 ±1.52	3.28 ±1.75
C-peptide ² , µg/d	74 ±38	86 ±40	75 ±42
Postprandial variables after lunch			
iAUC insulin, µU/ml x 2h	-	211 ±74	144 ±74 ^{**}
iAUC glucose, mg/dl x 2h	-	114 ±41	62 ±40 ^{***}
HOMA _{pp}	-	59 ±44	27 ±23 ^{**}

¹Values are means ± SDs; n = 17. ^{**}*P* < 0.01 (Wilcoxon's *t* test); ^{***}*p* < 0.001 (paired *t* test). BSD, breakfast-skipping day; DSD, dinner-skipping day; HOMA_{pp}, postprandial homeostasis model assessment; iAUC, incremental AUC; MAGE, mean amplitude of glycemic excursions; tAUC, total AUC.

²Repeated-measures ANOVA with Bonferroni adjustments was used.

Discussion

The present study aimed to investigate the impact of meal skipping on energy balance and metabolic risk. It was hypothesized, that breakfast skipping has an adverse impact on the regulation of energy balance and metabolic health compared to dinner skipping.

In the present study, the timing of meal skipping was important for inducing a change in macronutrient partitioning and energy expenditure. Breakfast skipping, on the one hand, increased 24-h fat oxidation and resulted in a corresponding negative fat balance, but dinner skipping, on the other hand, increased TEE.

In line with our primary hypothesis, only dinner skipping led to a small but significantly increased TEE (+69 kcal/d) compared with a conventional 3 meal pattern and thus improved energy balance under conditions of fixed energy intake. In accordance with the current study, a previous controlled metabolic chamber study found no effect of breakfast skipping on energy expenditure compared to a high feeding frequency of 7 meals. The study, however, showed that a low meal frequency of two meals (lunch and dinner) resulted in elevated energy expenditure during the postprandial hours, indicating a greater contribution of DIT to TEE [42]. In Addition, a higher DIT was found after consuming a standardized meal in the morning compared with the evening [48]. Therefore, the higher TEE on the DSD could be attributed to a higher 24-h DIT (in % of energy intake), when a large breakfast is consumed at a 2 meal/d pattern compared to smaller more frequent meals (control day). In contrast, a large dinner on the BSD does not seem to be able to increase the 24-h DIT sufficiently to result in a higher TEE compared to a 3-meal pattern.

This is supported by two further controlled studies utilizing metabolic chambers that found no effect of breakfast skipping on energy expenditure compared with a conventional 3 meal pattern [44] or a high feeding frequency of 6 meals [20]. This appears despite methodological differences between the studies. Taylor and Garrow [20] examined overweight and obese subjects under negative energy balance, Kobayashi et al. [44] investigated a small number of eight participants who had higher daylong glycemia with breakfast skipping and in the study by Verboeket-van de Venne and Westerterp [42] RQ and energy expenditure were calculated over 3-h intervals only. In Addition, in both

latter studies breakfast skipping was found to increase fat oxidation during the prolonged fasting period until the first meal at 1200.

Both breakfast and dinner skipping led to a longer duration of the overnight fasting period. Prolonged fasting can be considered as state of stress that leads to increased adrenergic activity and thus to a higher lipolysis and increased energy expenditure [136]. In line with this finding, levels of FFA (Table 2) were higher at both skipping days, whereas TEE was only higher with dinner skipping. Twenty-four-hour excretion of adrenaline and norepinephrine were however similar between both intervention days and the 3-meal control day. Nevertheless, we found that norepinephrine excretion inversely correlated with npRQ and positively with TEE when data from all intervention days were combined. The individual propensity of meal skipping to raise norepinephrine levels could, therefore, explain the inter-individual variance in fat oxidation and energy expenditure.

In addition to the duration of fasting, the timing of energy intake could also impact autonomic function and thus affect diurnal changes in substrate partitioning and energy expenditure. In line with this assumption, later timing of breakfast and dinner has been found to cause a phase delay in the diurnal 24-hour rhythm of cardiac autonomic nervous system activity assessed by HRV [137]. Although autonomic regulation assessed by heart rate monitoring differed with breakfast skipping and dinner skipping (Table 2), a higher SDNN on the BSD argues against a higher sympathetic tone and rather suggests improved autonomic regulation with breakfast skipping.

Lower 24-h insulin secretion due to a prolonged fasting period with meal skipping could contribute to increased lipolysis-induced fat oxidation. However, although levels of FFA were higher with both meals skipping days when compared with the three-meal control day, 24-h insulin secretion did not differ between all intervention days (Table 3). Of note, insulin excursions rather than cumulative 24-h insulin secretion are more important for the regulation of nutrient partitioning. Although a high frequency of six, compared to three meals, was associated with lower daylong insulin AUC, at the same time it caused a marked suppression in daylong FFA concentrations between meals. This was due to the fact that frequent eating prevents a drop in insulin that facilitates lipolysis [43].

A limitation of the study protocol is that the effects of meal skipping on voluntary energy intake cannot be examined. Although ghrelin levels were higher in the morning

with breakfast skipping and in the evening with dinner skipping, we found no differences in the AUC of daylong ghrelin levels between meal skipping days and three meal control (Figure 4). However, a compensation of a higher energy expenditure and fat oxidation by a higher spontaneous energy or fat intake under *ad libitum* conditions cannot be ruled out. Interestingly, a higher meal frequency of 14 or 6 compared to 3 meals led to increased ghrelin levels [21], and ratings of hunger and "desire to eat" [21,43]. In Addition, extending morning fasting until lunch caused incomplete energy compensation with an *ad libitum* lunch [138,139]. Increased hunger and decreased satiety in response to breakfast skipping were found primarily in habitual breakfast eaters [140]. This may suggest that the effect of meal skipping on appetite regulatory systems is enhanced in habitual breakfast eaters. In the present study, no differences were observed between regular breakfast eaters and occasional breakfast skippers on meal skipping-induced changes in ghrelin levels, glucose regulation and TEE or fat oxidation (data not shown).

An additional limitation of the present study is the fact that only responses to the first day of breakfast skipping or dinner skipping were measured and therefore the metabolic consequences of habitual breakfast skipping or dinner skipping remain unclear.

In patients with type 2 diabetes, habitual breakfast skipping was associated with a later chronotype that contributed to poorer glycemic control [141]. A disrupted circadian clock provides a mechanistic explanation for the relationship between a disturbed diurnal eating pattern and alterations in glucose metabolism [142]. Glucose metabolism is highly circadian [143] and depends largely on the timing and composition of nutrient ingestion. Because the body uses nutrient input to set circadian rhythms [144], it is possible that both timing and nutrient composition of the diet might be important for prevention of metabolic disturbances. In line with impaired metabolic function with breakfast skipping, randomized controlled trials support higher glucose variability in lean subjects and impaired insulin sensitivity in obese participants with breakfast skipping when compared with breakfast eating but found no effect on body weight or fat mass over a six-week period [145,146].

In support of impaired glucose homeostasis with breakfast skipping, postprandial HOMA index and glucose levels after lunch were higher on the BSD compared to the DSD (Table 3). Compared with the DSD, higher postprandial fat oxidation at lunchtime after breakfast skipping occurred despite increased insulin levels and suggests metabolic

inflexibility after prolonged fasting. Mitochondrial capacity to switch freely between oxidative fuels in the transition from fasting to feeding is, therefore, lost [147]. In a healthy, metabolically flexible state, consumption of a high-carbohydrate meal results in a rise in blood insulin levels and respiratory quotient, indicative of a robust shift from fatty acid to glucose oxidation. Increased fat oxidation, despite higher postprandial insulin concentrations with breakfast skipping, suggest the development of metabolic inflexibility in response to prolonged fasting that may increase metabolic risk over time.

Altogether, the present results support the association between breakfast skipping and disturbed glucose homeostasis, which is not explained by a positive energy balance. On the contrary, breakfast skipping increased 24-h fat oxidation and dinner skipping increased total energy expenditure. In conclusion, a causal role of breakfast skipping for the development of obesity is not supported by the present data.

Chapter IV

***Impact of daylong changes in energy flux on energy and
macronutrient metabolism during energy balance, caloric
restriction and overfeeding***

Subjects and Methods

Sixteen healthy adults (3 women and 13 men) were recruited by notice board postings at the University of Hohenheim and on the social media platform Facebook between December 2016 and February 2018 and were included in the study. Exclusion criteria were food allergies or intolerances, alternative nutrition habits, competitive sports, smoking, chronic diseases or regular use of medications. The study protocol was approved by the ethics committee of the Medical Council of Baden-Württemberg, Germany. The trial was registered at clinicaltrials.gov as NCT03361566. All subjects provided written informed consent before participation.

Study protocol

The randomized crossover trial was conducted at the University of Hohenheim. An outline of the study protocol is given in **Figure 1**. The participants underwent 24-h interventions in a caloric chamber with 3 different levels of energy flux (EF): (i) low, physical activity level (PAL) = 1.3 - 1.4 (ii) medium, PAL = 1.5 - 1.6 and (iii) high, PAL = 1.7 - 1.8. Each EF level was carried out at energy balance (EB), caloric restriction (CR) and overfeeding (OF) (100%, 75% or 125% of individual energy requirement). Thus, in total, the study protocol consists of 9 24-h intervention days. Different levels of EF were accomplished by walking on a treadmill (Kettler Track 9, KETTLER GmbH, Ense, Germany) with 4 km/h for different time periods. During low EF participants were required to stay sedentary. During medium EF, they walked for 3 x 55 min and during high EF for 3 x 110 min. The three interventions with different EF levels were separated by one washout-day and the three energy balance conditions were also separated by at least one washout-day to avoid any carry-over effects. A three-day run-in period with controlled diet preceded the intervention phase.

Low, medium and high EF levels as well as OF and CR were randomized by block randomization. Therefore, the sequence of the three energy balance conditions was either CR-EB-OF or OF-EB-CR.

The 24h-interventions took place between 0600 in the morning to 0600 the following morning. Participants were admitted to the institute at 1830 on the day before the 24-h

intervention to spend the night before the intervention in the metabolic chamber. Participants left the morning after the intervention day. Therefore, in total participants spent almost 36 h in the metabolic chamber for one intervention day. On the washout day, they were allowed to go home for 12 h. During the interventions in the caloric chamber, participants followed a constant daily routine: wake up at 0600; meals at 0700, 1300, 1900; and bedtime at 2230. Prescribed physical activity at medium and high EF was performed after each meal at 0740, 1340 and 1940. After waking up, participants left the metabolic chamber for a few minutes between 0630 and 0645 to place an intravenous catheter. Blood samples were taken every 2 h during the intervention-days between 0700 and 2100.

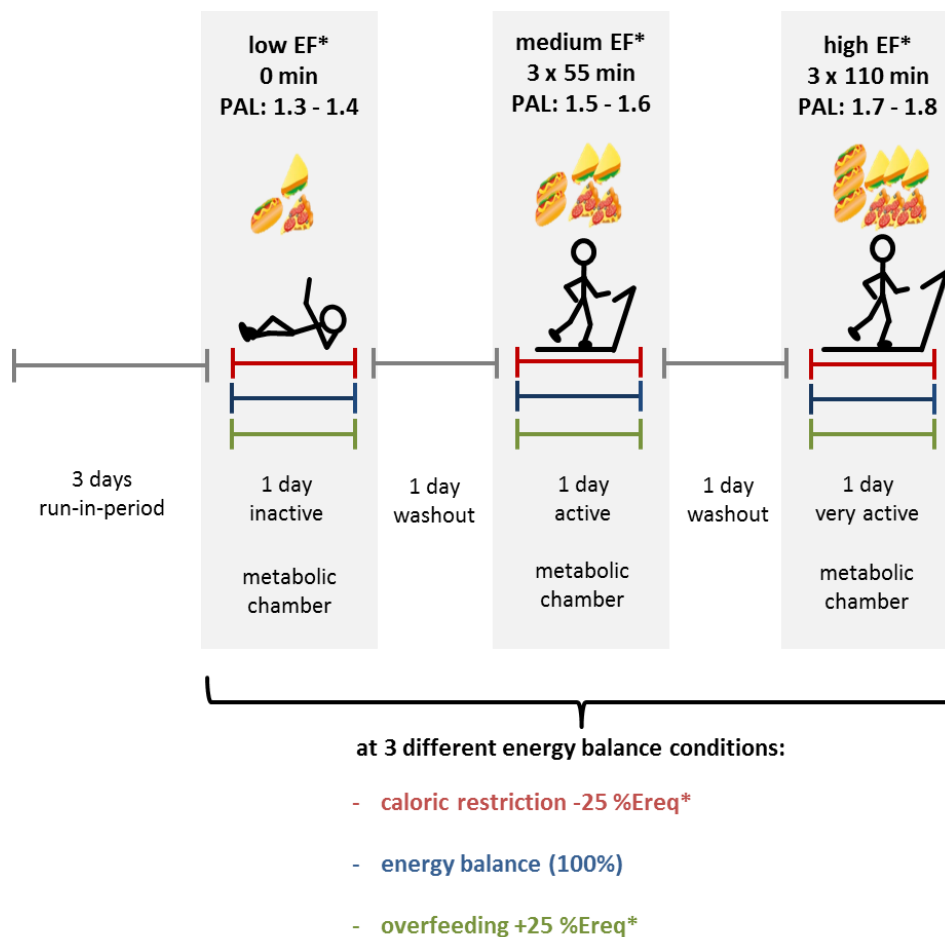


Figure 1 Outline of the study protocol of a randomized crossover trial with 24-h interventions in a metabolic chamber with 3 different levels of energy flux: low, medium, and high; each at energy balance, caloric restriction and overfeeding (100%, 75% or 125% of individual energy requirement). Different levels of EF were accomplished by walking on a treadmill with 4 km/h for various time periods (0, 3 x 55 min, 3 x 110 min). A 3-day run-in period with a controlled diet preceded the intervention phase and EF level interventions were separated by one washout-day.

*Randomly assigned. EF, energy flux; PAL, physical activity level; Ereq, energy requirement

Control of energy intake

All foods during the study period were provided from the metabolic kitchen at the Institute of Nutritional Medicine. Participants were instructed to only consume the provided food and to only drink water and unsweetened herbal or fruit tea. Throughout the whole study period, macronutrient composition was kept constant with 50% carbohydrate, 35% fat and 15% protein for each day and each meal. During the 24-h interventions, participants received the same food items on each day and were asked to eat all the provided food within half an hour. Individual energy intake was based on individual energy requirement. Therefore, in advance of the study individual energy expenditure for each EF was measured by 24-h room calorimetry using the same procedure as on the 24-h interventions (pre-study test). On washout-days, during the 3-day run-in period and during the pre-study test, participants ate *ad libitum* and leftovers were back-weighed to calculate dietary intake. Under the condition of caloric restriction energy intake was calculated to be 25% less than the energy requirement for the respective EF level and under the condition of overfeeding 25% greater. Individual diet composition was calculated by using Prodi®6 software (Wissenschaftliche Verlagsgesellschaft, Stuttgart, Germany).

Control of physical activity

In advance of the study, it was tested which walking time and walking speed were appropriate to reach the predetermined PALs. The walking speed was set at 4 km/h to mimic low intensity everyday physical activity. On medium EF the participants had to walk 165 (3 x 55) minutes and on high EF 330 (3 x 110) minutes. Hence, they covered a distance of 11 km during medium and 22 km during high EF. Walking time, distance and speed were controlled with the software Kettler World Tours 2.0 (KETTLER GmbH, Ense, Germany).

Participants were asked to stay sedentary and to spend their time sitting at the desk or lying in bed during all interventions except for the walking session at medium and high EF. However, they were not allowed to sleep during the day. On the washout-days and

during the three-day run-in period, participants were asked to refrain from physical exercise to avoid any impact of such activity on the outcome parameters [148,149].

Throughout the entire study period, the step count per hour was continuously measured by using a triaxial activity monitor (ActivPAL, Paltechnologies Ltd., Glasgow, UK). The data were analyzed with the Software activPAL Professional v7.2.32. The ActivPAL was worn at the mid-line of the thigh, one-third of the way between hip and knee fixed with a waterproof tape according to the recommendation of the manufacturer. The ActivPAL data of the participants were only included in the analysis when energy expenditure data were also available for the respective participant. This was done in order to have consistent datasets for energy expenditure and physical activity data. Because of technical problems with the ActivPAL device, the data of one participant are missing.

Anthropometry and body-composition analysis

Examination of baseline anthropometry and body composition took place before to the 3-day run-in period of the pre-study test after an overnight fast. Height was measured with a stadiometer (Seca 274, Seca GmbH&Co.KG, Hamburg, Germany). Body weight was measured on a calibrated impedance scale (Seca mBCA 515, Seca GmbH&Co.KG, Hamburg, Germany). Fat mass (FM) was assessed using Air Displacement Plethysmography (ADP) via the BodPod Body Composition System (COSMED, Rome, Italy). Fat mass index (FMI) was calculated as FM divided by the square of height (kg/m^2). Fat-free mass index (FFMI) was calculated as FFM divided by the square of height (kg/m^2).

Energy expenditure and macronutrient oxidation

The metabolic chambers and the assessment of TEE, 24h-macronutrient oxidation, SEE and DIT were described in Chapter II. The FR of the metabolic chambers was determined as 120 l/min. Relative energy (rE_B) and macronutrient balance (%) were calculated as percent energy intake (E_i) of respective TEE ($\frac{E_i}{\text{TEE}} \times 100$) and as percent 24-

macronutrient oxidation of respective macronutrient intake ($\frac{24\text{-h oxidation}}{\text{intake}} \times 100$). In order to examine macronutrient utilization (fuel partitioning) percent macronutrient oxidation of TEE was calculated ($\frac{24\text{-h oxidation}}{\text{TEE}} \times 100$). Physical activity level was determined as TEE divided by REE (REE= SEE + SEE x 0.05). Energy expenditure during activity (EE_{active}, kcal/min) was determined for medium and high EF as the mean rate of energy expenditure when the participants were walking on the treadmill. Energy expenditure during inactivity (EE_{inactive}, kcal/min) was determined for low, medium and high EF as the mean rate of energy expenditure between 1030-1230 and 1630-1830. DIT was calculated for low EF in a subsample of 7 participants. Energy expenditure data from 5 participants and macronutrient oxidation data from 7 participants were excluded from the final analysis because of problems in the data acquisition and processing procedure as described in Chapter II of this thesis.

Blood and urine parameters

Free fatty acids in serum were measured photometrically, and total area under the curve (tAUC) was calculated for 14 h (0700–2100). Epinephrine and norepinephrine excretions in 24-h urine were measured by using liquid chromatography-mass spectrometry. 24-h urine was acidified with hydrogen chloride within 6-7 h after the beginning of the 24-h sampling period.

Statistical analyses

Data are reported as means \pm SDs. The statistical software R (2017) was used to evaluate the data using an appropriate statistical mixed model [150,151]. The data were assumed to be normally distributed and to be heteroscedastic due to the different conditions of energy balance and EF level. These assumptions are based on a graphical residual analysis. The statistical model included the 3 energy balance conditions (EB, CR, OF) and the 3 EF levels (low, medium, high), as well as their interaction term as fixed factors. The ID was regarded as a random factor. The correlations of the measurement

values between several intervention days were taken into account (auto-correlation). Based on this model, a Pseudo R^2 was calculated [152] and an analysis of covariances (ANOVA) was conducted, followed by multiple contrast tests (e.g., see [153]) in order to compare the several levels of the influence factors, respectively. Comparisons were made between different levels of EF within the same energy balance condition as well as between different energy balance conditions within the same level of EF. For the three EF levels, all possible comparisons were considered (low to medium, low to high, medium to high). For the comparison of energy balance conditions, the equal energy balance (EB) was considered as control and the comparison between EB and CR as well as EB and OF, but not the comparison between CR and OF was analyzed. Deviations of the relative energy balances (%) from the values, that were predetermined by the study protocol (100, 75 and 125) and deviations of relative fat, carbohydrate, and protein balances (%) from 100 (intake = oxidation) were tested by one-sample t test. Correlations between the changes in SEE and the changes in epinephrine or norepinephrine (differences between low to medium and to high EF as well as between medium to high EF) were tested by Pearson's correlation coefficient for each energy balance condition.

Results

Baseline characteristics of the study population are shown in **Table 1**. Three women and 13 men aged 20-30 y participated in this study. BMI (in kg/m²) and percentage of body fat mass (%) ranged between 19.6 to 31.2 and 8.7 to 45.1, respectively. According to WHO criteria, five participants were overweight, and one was obese. For the analyses of energy metabolism data one woman and 10 men were included (Subsample 1). In this subsample BMI (in kg/m²) and percentage of body fat mass (%) ranged between 19.6 to 27.2 and 10.8 to 31, respectively. For the analyses of macronutrient metabolism data (Subsample 2), 9 men were included. In this subsample, BMI ranged between 19.6 to 26.1 kg/m². Body fat mass range is identical with subsample 1.

Table 1 Baseline characteristics of the total study population and the two subsamples¹

	All Subjects n = 16	Subsample 1 ² n = 11	Subsample 2 ³ n = 9
Age, y	25.1 ± 3.9	25.5 ± 4.0	26.6 ± 3.5
Height, m	1.77 ± 0.09	1.78 ± 0.08	1.79 ± 0.06
Body weight, kg	75.2 ± 11.7	74.1 ± 9.2	74.4 ± 10.1
BMI, kg/m ²	24.0 ± 3.2	23.4 ± 2.2	23.0 ± 2.1
Fat mass, %	21.2 ± 9.7	18.9 ± 7.3	18.3 ± 6.2
FMI, kg/m ²	5.3 ± 3.2	4.5 ± 2.2	4.3 ± 1.8
FFMI, kg/m ²	18.6 ± 1.4	18.8 ± 1.3	18.7 ± 1.3

¹ Values are means ±SDs. FMI, fat mass index; FFMI, fat-free mass index

² Subjects included in the analyses of energy expenditure variables

³ Subjects included in the analyses of macronutrient oxidation variables

Comparisons of energy metabolism parameters and physical activity between conditions of energy balance and between EF levels are presented in **Table 2**. As predetermined by study design, energy intake (E_i , kcal/d) differs between the 3 EF levels and between the energy balance conditions and TEE (kcal/d) increased with higher EF level. **Figure 2** demonstrates the daylong profiles of TEE for all intervention days. Furthermore, E_i was calculated to match TEE during EB. This was achieved at medium and high EF ($rE_B = 100\%$, $p > 0.05$), but at low EF, E_i was slightly higher than TEE (+75 kcal, $p < 0.05$). As expected, diet-induced thermogenesis (kcal/d) at low EF was decreased during CR and increased during OF compared to EB (Table 2). There was no difference in DIT as a percentage of energy intake between the energy balance conditions. Daily step count (steps/d) increased with higher EF within all energy balance conditions (Table 2). There was no difference in step count at the same EF level between the three energy balance conditions. PAL at the same EF did not differ between the energy balance conditions but was different by design between the EF levels within each energy balance condition.

Components of macronutrient metabolism are shown in **Table 3**. Fat-, carbohydrate and protein intake (F_i , CHO_i , P_i ; g/d) increased with higher EF and differ between the energy balance conditions as predetermined by study design.

Impact of EF on the regulation of energy balance

Sleeping energy expenditure (kcal/3h) showed an overall interaction between energy balance condition and EF level ($p < 0.05$). SEE increased at medium and high EF compared to low EF during EB and OF, but not during CR (**Figure 3**).

Relative energy balance during CR and OF did not differ between the EF levels (Table 2). During CR and OF, E_i was calculated to be 25% lower and 25% higher than TEE. Actual rE_B during CR was higher than 75% at all EF levels ($p < 0.05$). During OF, rE_B did not differ from the expected value of 125% at all EF levels.

Energy expenditure during physical activity (kcal/min) was increased at high EF compared to medium EF during CR and OF, but not during EB (Table 2). Energy

expenditure during inactivity (g/min) was increased at medium and high EF compared to low EF at EB and OF, but not at CR (Table 2).

The ability of an increased EF to prevent a decrease in energy expenditure with caloric restriction or to promote energy dissipation during overfeeding

An overall interaction between energy balance condition and EF level was observed for TEE ($p < 0.05$). When comparing TEE and SEE of the same EF level between CR and EB, a decrease during CR was observed only at medium EF (Table2). At medium EF, EE_{active} was lower during CR compared to EB, however not with high EF. Energy expenditure during inactivity at medium EF was lower during CR compared to EB and there was a tendency for a lower $EE_{inactive}$ at high EF.

There was no difference in TEE, SEE, EE_{active} and $EE_{inactive}$ at the same EF level during OF and EB.

Table 2 Comparison of energy expenditure variables and physical activity between conditions of energy balance and between the EF levels¹

	energy balance			caloric restriction			overfeeding		
	low EF	medium EF	high EF	low EF	medium EF	high EF	low EF	medium EF	high EF
E_i (kcal/d)	2378 ± 311	2845 ± 383 ^a	3278 ± 489 ^{a, b}	1784 ± 233 ^c	2151 ± 291 ^{a, c}	2458 ± 390 ^{a, b, c}	2974 ± 387 ^c	3584 ± 484 ^{a, c}	4085 ± 628 ^{a, b, c}
TEE (kcal)	2303 ± 263	2855 ± 302 ^a	3235 ± 419 ^{a, b}	2259 ± 339	2684 ± 379 ^{a, c}	3121 ± 448 ^{a, b}	2340 ± 306	2861 ± 366 ^a	3296 ± 437 ^{a, b}
rE_B (%)	103 ± 4	99 ± 4 ^a	101 ± 6	79 ± 6 ^c	80 ± 4 ^c	79 ± 3 ^c	127 ± 6 ^c	125 ± 6 ^c	124 ± 6 ^c
DIT ² (kcal/24h)	386 ± 84	-	-	296 ± 80 ^c	-	-	442 ± 88	-	-
DIT ² (%)	17 ± 3	-	-	17 ± 4	-	-	16 ± 2	-	-
Steps ³	415 ± 189	17762 ± 1275 ^a	34920 ± 2018 ^{a, b}	482 ± 218	17702 ± 1240 ^a	34859 ± 2459 ^{a, b}	463 ± 234	17806 ± 1379 ^a	34937 ± 2350 ^{a, b}
PAL	1.30 ± 0.04	1.57 ± 0.05 ^a	1.77 ± 0.09 ^{a, b}	1.27 ± 0.06	1.54 ± 0.10 ^a	1.74 ± 0.09 ^{a, b}	1.35 ± 0.06	1.55 ± 0.08 ^a	1.75 ± 0.10 ^{a, b}
EE_{active} (kcal/min)	-	3.76 ± 0.45	3.86 ± 0.50	-	3.58 ± 0.58 ^c	3.75 ± 0.60 ^b	-	3.74 ± 0.61	3.86 ± 0.63 ^b
$EE_{inactive}$ (kcal/min)	1.63 ± 0.19	1.77 ± 0.25 ^a	1.77 ± 0.27 ^a	1.57 ± 0.26	1.62 ± 0.22 ^c	1.65 ± 0.29 ^t	1.68 ± 0.25	1.77 ± 0.26 ^a	1.86 ± 0.31 ^a

¹ Values are means ± SDs; n = 11. ^ap<0.05 for comparison with low EF within the energy balance condition, ^bp<0.05 for comparison with medium EF within the energy balance condition, ^cp<0.05 for comparison of the same EF level with energy balance, ^tp=0.056 for comparison of the same EF level with energy balance. Multiple contrast tests. EF, energy flux; E_i , energy intake; TEE, total energy expenditure; rE_B , relative energy balance; DIT, diet-induced thermogenesis; PAL, physical activity level; EE_{active} , energy expenditure during activity; $EE_{inactive}$, energy expenditure during inactivity;

² n = 7

³ n = 8

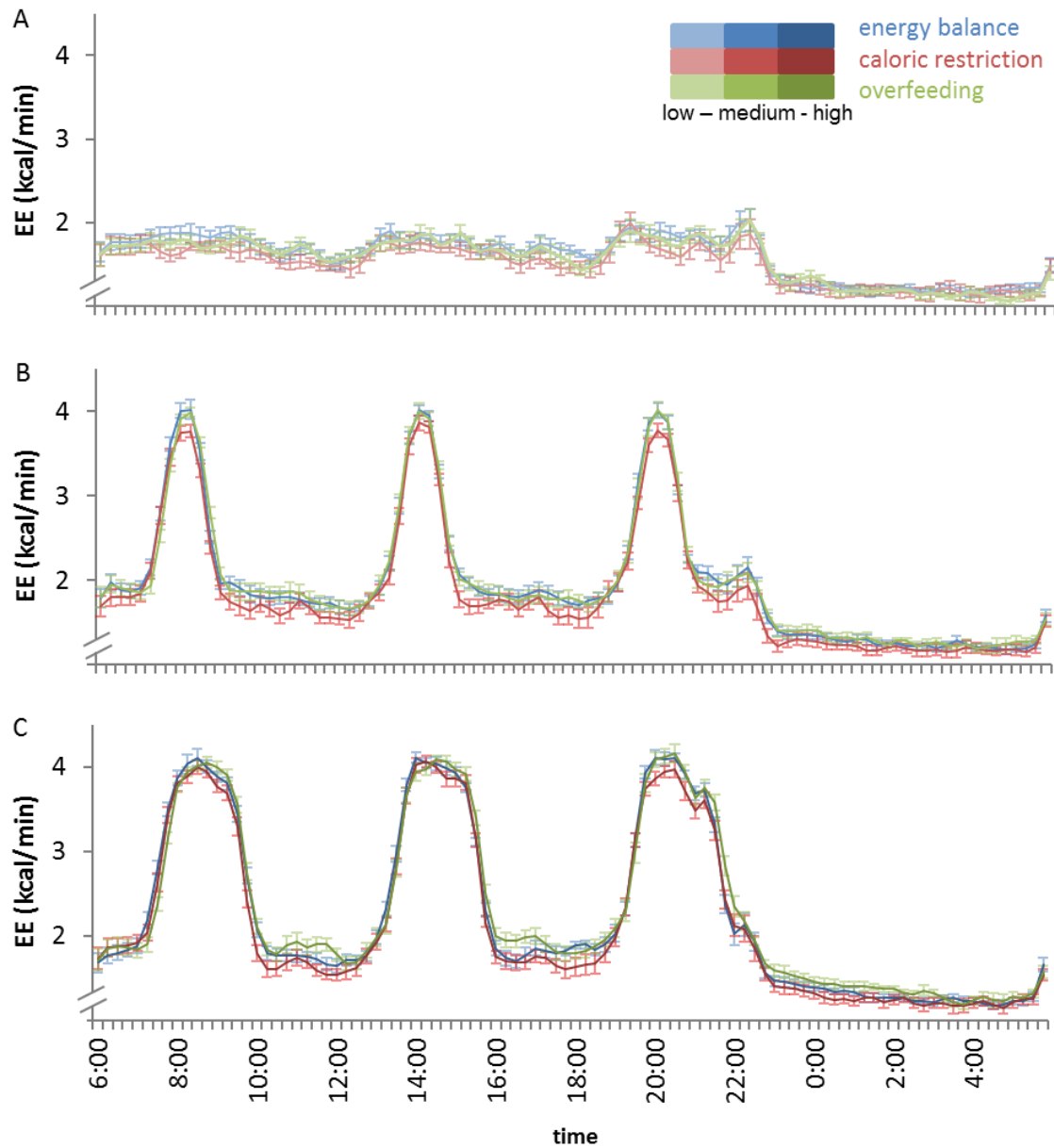


Figure 2 Comparison of the daylong profile of energy expenditure between the three conditions of energy balance at low EF (A) medium EF (B) and high EF (C) ($n = 11$). Mean values are shown for 15 min intervals and SEs only every 30 min for clarity. Differences in the corresponding TEE are reported in Table 2. EF, energy flux; EE, energy expenditure.

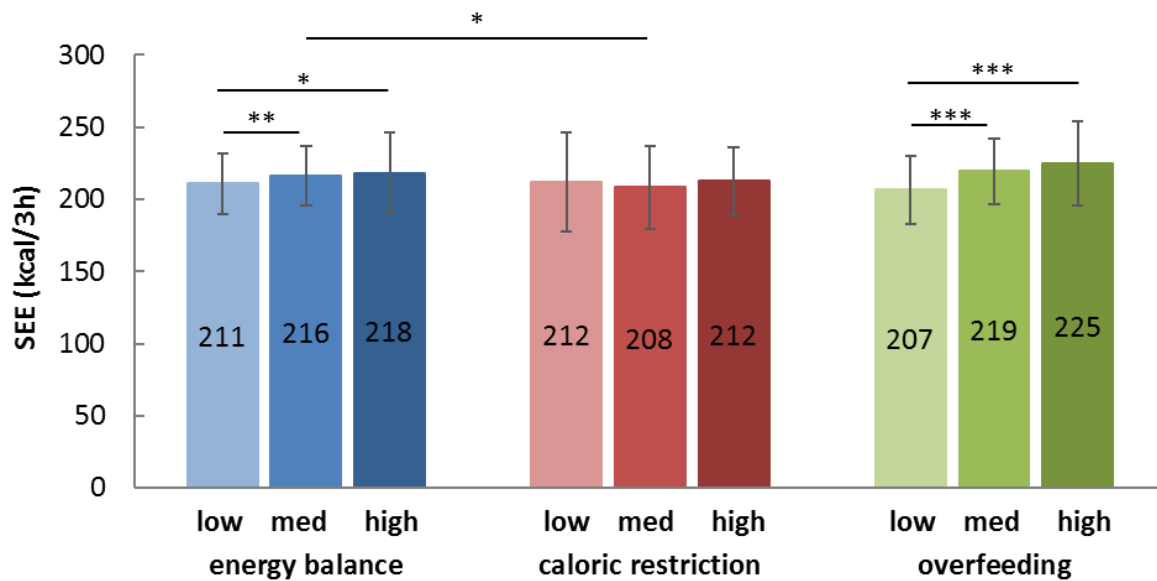


Figure 3 Comparison of sleeping energy expenditure ($n = 11$) between different levels of energy flux and between different levels of energy balance. Values are means \pm SDs. Multiple contrast tests; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. SEE, sleeping energy expenditure

Impact of energy flux on fat oxidation

Figure 4 demonstrates the 24-h fat oxidation profile during all intervention days. Twenty-four-hour fat oxidation (24-h F_{OX} , g/d) increased with higher EF during EB and OF (**Table 3**). During CR, 24-h F_{OX} was higher at high compared to low EF, but this was not the case at medium EF and there was no difference in 24-h F_{OX} between medium and high EF.

During EB and OF, fat intake (F_I) was higher than 24-h F_{OX} at all EF levels (relative fat balance, $rF_B > 100\%$, $p < 0.05$), but rF_B was higher at medium and high EF compared to low EF (**Table 3**). During CR, F_I equals 24-h F_{OX} at all EF levels ($rF_B = 100\%$, $p > 0.05$) and there was no difference in rF_B between EF levels.

Fat oxidation as a percentage of TEE (F_{OX}/TEE ; %) was higher at high EF compared to low EF during EB and OF and at medium EF during OF. During CR, F_{OX}/TEE did not differ between all EF levels (**Table 3**). Total area under the curve for free fatty acids ($\text{mg/dl} \times 14\text{h}$) was increased at medium EF compared to low EF during EB and OF and at high EF during OF. There was no difference in FFA_{tAUC} between the EF levels during CR (**Table 3**).

The ability of an increased energy flux to prevent a decrease in fat oxidation with OF or to further promote fat oxidation during CR

During CR, 24-h F_{OX} was higher compared to EB only at low EF, but not at medium or high EF. There was no difference in 24-h F_{OX} at the same EF level between EB and OF (Table 3).

During CR, F_{OX}/TEE was only higher at low EF compared to EB. During OF, F_{OX}/TEE was lower at medium and high EF compared to EB, but not at low EF. There was an overall interaction between energy balance condition and EF level for F_{OX}/TEE ($p < 0.05$).

During CR, FFA_{tAUC} was higher at all EF levels compared to EB. No difference in FFA_{tAUC} was observed between EB and OF.

Impact of energy flux on carbohydrate oxidation

Twenty-four-hour carbohydrate oxidation (24-h CHO_{OX} ; g/d) increased with increasing EF during EB, CR and OF (Table 3). There was no difference in relative carbohydrate oxidation ($rCHO_B$, %) between the EF levels at all energy balance conditions (Table 3).

Twenty-four-hour carbohydrate oxidation was higher than CHO_I ($rCHO_B > 100\%$, $p < 0.05$) at all EF levels during EB and CR and at low EF level during OF. During OF, 24-h CHO_{OX} was equal to CHO_I at medium and high EF level ($rCHO_B = 100\%$, $p > 0.05$).

Carbohydrate oxidation as a percentage of TEE (CHO_{OX}/TEE ; %) did not differ between EF levels during EB, CR and OF (Table 3).

Impact of energy flux on protein oxidation

Twenty-four-hour protein oxidation (24-h P_{OX} ; g/d) did not differ between EF levels during EB and CR, but there was a tendency towards a higher 24-h P_{OX} with high EF compared to low EF during EB (Table 3). During OF, 24-h P_{OX} was higher at medium and high EF compared to low EF.

Relative protein balance (rP_B ; %) decreased with increased EF level at all energy balance conditions (Table 3). During energy balance, 24-h P_{OX} was higher than P_I ($rP_B > 100\%$, $p < 0.05$) at low EF, equal ($rP_B = 100\%$, $p > 0.05$) at medium EF and lower ($rP_B < 100\%$, $p < 0.05$) at high EF. During CR, 24-h P_{OX} was higher than P_I at all EF levels ($rP_B > 100\%$, $p < 0.05$). During OF, 24h- P_{OX} was lower than P_I at all EF levels ($rP_B > 100\%$, $p < 0.05$).

Protein oxidation as a percentage of TEE (P_{OX}/TEE ; %) was increased at medium and high EF compared to low EF during EB and OF and at high EF compared to medium EF during EB. During CR, no difference was observed in P_{OX}/TEE between EF levels (Table 3). There was an overall interaction between energy balance condition and EF level for rP_B and P_{OX}/TEE ($p < 0.05$).

Table 3 Comparison of macronutrient intake and oxidation variables between conditions of energy balance and between the EF levels¹

	energy balance			caloric restriction			overfeeding		
	low EF	medium EF	high EF	low EF	medium EF	high EF	low EF	medium EF	high EF
F_I (g/d)	95 ±12	114 ±15 ^a	132 ±20 ^{a, b}	72 ±9 ^c	86 ±11 ^{a, c}	98 ±17 ^{a, b, c}	119 ±15 ^c	144 ±18 ^{a, c}	165 ±25 ^{a, b, c}
24h-F_{OX} (g/d)	51 ±14	80 ±12 ^a	102 ±27 ^{a, b}	78 ±25 ^c	93 ±35	107 ±28 ^a	41 ±11	71 ±20 ^a	86 ±20 ^{a, b}
rF_B (%)	54 ±15	71 ±7 ^a	77 ±13 ^a	108 ±24 ^c	105 ±28 ^c	109 ±19 ^c	35 ±10 ^c	49 ±10 ^{a, c}	52 ±6 ^{a, c}
F_{OX}/TEE (%)	20 ±5	26 ±2	28 ±5 ^a	31 ±6 ^c	31 ±8	31 ±5	16 ±4	22 ±4 ^{a, c}	24 ±3 ^{a, c}
FFA_{TAUC}² (mg/dl*14h)	87 ±22	94 ±22 ^a	99 ±32	112 ±22 ^c	115 ±24 ^c	116 ±20 ^c	78 ±23	91 ±25 ^a	92 ±27 ^a
CHO_I (g/d)	296 ±39	356 ±48 ^a	408 ±61 ^{a, b}	223 ±29 ^c	269 ±36 ^{a, c}	309 ±48 ^{a, b, c}	372 ±48 ^c	448 ±60 ^{a, c}	512 ±78 ^{a, b, c}
24h-CHO_{OX} (g/d)	378 ±55	451 ±45 ^a	500 ±39 ^{a, b}	304 ±43 ^c	378 ±42 ^{a, c}	456 ±55 ^{a, b}	409 ±60	474 ±52 ^{a, c}	541 ±71 ^{a, b, t1}
rCHO_B (%)	128 ±13	127 ±9	124 ±14	137 ±19	142 ±20	149 ±10 ^c	110 ±9 ^c	106 ±9 ^c	106 ±10 ^c
CHO_{OX}/TEE (%)	66 ±6	64 ±2	62 ±5	54 ±6 ^c	58 ±8	59 ±5	70 ±5	67 ±4 ^c	66 ±4
P_I² (g/d)	88 ±12	106 ±15 ^a	120 ±19 ^{a, b}	66 ±9 ^c	79 ±11 ^{a, c}	92 ±15 ^{a, b, c}	110 ±15 ^c	131 ±18 ^{a, c}	152 ±25 ^{a, b, c}
24h-P_{OX}² (g/d)	100 ±15	104 ±19	105 ±16 ^{t2}	96 ±16	97 ±17	100 ±13	104 ±14	111 ±15 ^a	116 ±18 ^{a, c}
rP_B² (%)	113 ±7	98 ±11 ^a	88 ±10 ^{a, b}	146 ±11 ^c	122 ±11 ^{a, c}	109 ±8 ^{a, b, c}	95 ±8 ^c	85 ±9 ^{a, c}	77 ±10 ^{a, b, c}
P_{OX}/TEE (%)	18 ±1	15 ±1 ^a	13 ±1 ^{a, b}	14 ±4 ^c	13 ±2	12 ±1	18 ±2	15 ±1 ^a	14 ±2 ^a

¹Values are means ± SDs; n = 9. ^ap<0.05 for comparison with low EF within the energy balance condition, ^bp<0.05 for comparison with medium EF within the energy balance condition, ^cp<0.05 for comparison of the same EF level with energy balance, ^{t1}p=0.056 for comparison of the same EF level with energy balance. ^{t2}p=0.059 for comparison with low EF within the energy balance condition. Multiple contrast tests. F_I, fat intake; F_{OX}, fat oxidation; rF_B, relative fat balance; F_{OX}/TEE, percent fat oxidation of total energy expenditure; CHO_I, carbohydrate intake; CHO_{OX}, carbohydrate oxidation; rCHO_B, relative carbohydrate balance; CHO_{OX}/TEE, percent carbohydrate oxidation of total energy expenditure; P_I, protein intake; P_{OX}, protein oxidation; rP_B, relative protein balance; P_{OX}/TEE, percent protein oxidation of total energy expenditure

²n=16

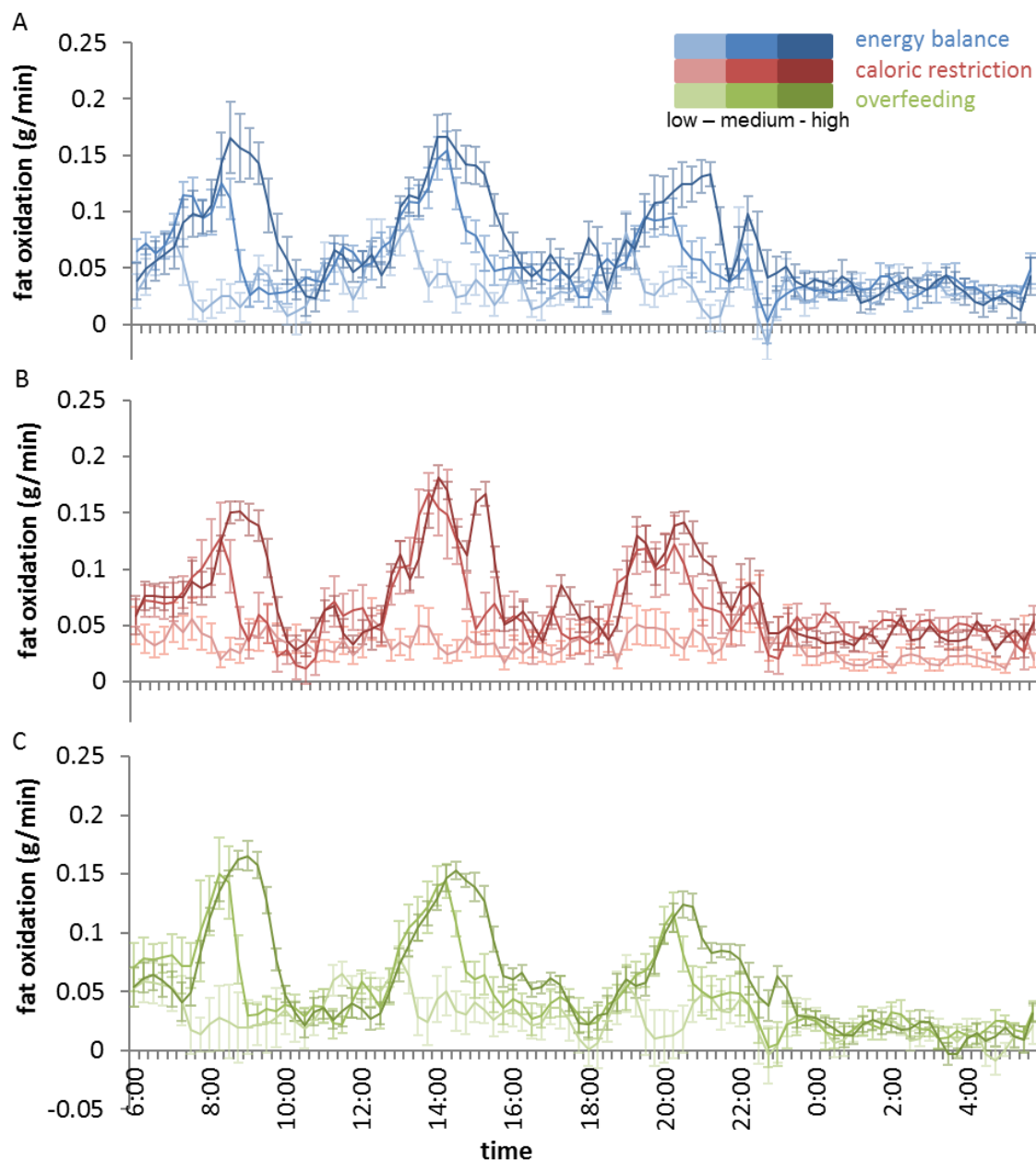


Figure 4 Comparison of fat oxidation between the 3 EF levels at energy balance (A) caloric restriction (B) and overfeeding (C) ($n = 9$). Mean values are shown for 15 min intervals and SEs only at every 30 min for clarity. Differences in the corresponding 24-h fat oxidation are reported in Table 3.

Catecholamines

There was no difference in adrenaline and norepinephrine excretion between the EF levels at all energy balance conditions (data not shown, all $p > 0.05$). Changes in sleeping energy expenditure between the EF level and changes in norepinephrine excretion between the EF levels (differences between low to medium and to high EF as well as between medium to high EF) showed a positive correlation during EB ($r = 0.43$, $p = 0.013$) and OF ($r = 0.45$, $p = 0.007$) and therefore indicate, that an increase in SEE was associated with an increase in norepinephrine concentration (**Figure 5**). There was no association between the changes in SEE and the changes in norepinephrine excretion during CR ($p > 0.05$). In addition, no correlation was found between changes in SEE and changes in epinephrine excretion during all energy balance conditions (all, $p > 0.05$).

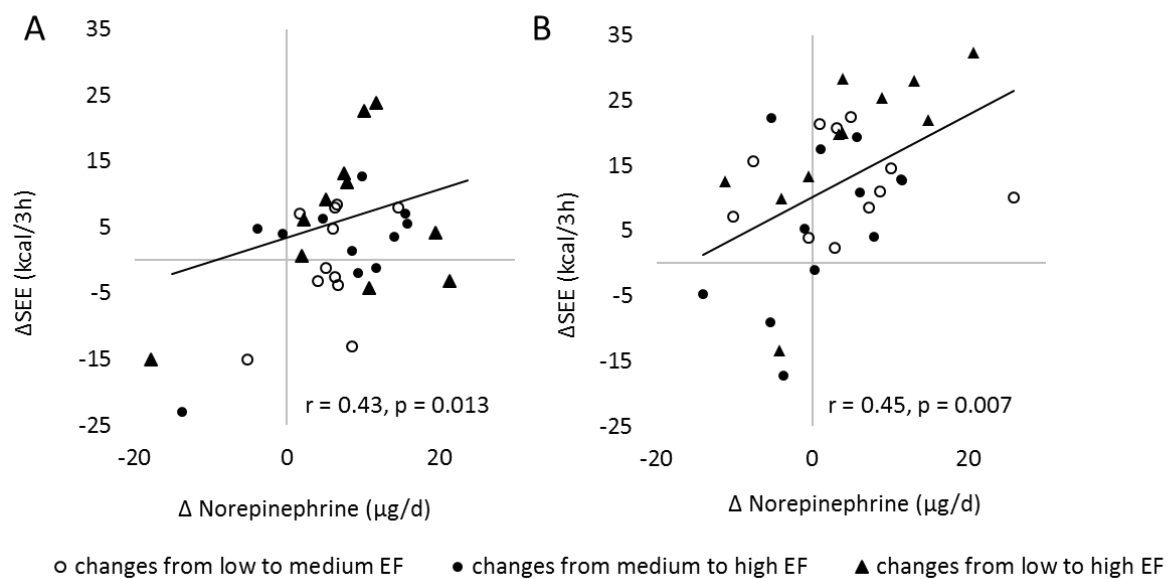


Figure 5 Association between changes in sleeping energy expenditure and changes in norepinephrine excretion between the EF levels during energy balance (A) and overfeeding (B). Correlations include data of all intervention days ($n = 11$). Pearson's correlation coefficient. SEE, sleeping energy expenditure; EF, energy flux

Discussion

The present study aimed to investigate the impact of EF on energy expenditure and fat oxidation during equal energy balance, caloric restriction and overfeeding. It was hypothesized, that the regulation of energy balance and fat balance is improved with higher EF.

The main findings of this study were a higher SEE and improved regulation of fat balance at a higher energy flux during energy balance and overfeeding, whereas a high energy flux had no impact on these outcome parameters during caloric restriction. In the following, the study results are discussed. First, the impact of EF on energy expenditure and then the impact of EF on macronutrient oxidation with a focus on fat oxidation is discussed.

Impact of energy flux on energy expenditure

In line with the proposed hypothesis, a medium and high EF led to a higher SEE during EB (medium EF: +44 kcal/d, high EF: +58 kcal/d) and OF (medium EF: +100 kcal/d, high EF: +144 kcal/d) compared to a low EF (Figure 3). Consistently, other studies have shown increased REE values with a higher EF level [52,53,154,155]. Bullough et al. investigated eight trained men in a cross-over design, which included the comparison of three days in energy balance with either high EF or low EF [154]. Increased mean REE values (about +190 kcal/d) were observed at high EF compared to low EF. In this study, the high EF state was reached with 90 min of intensive exercise training (75% VO_2max) on a bicycle ergometer. Goran et al. compared mean REE values of five healthy young men, who underwent a low EF state for 10 days to a group of six men in a high EF state (180 min exercise with 50% VO_2max) during energy balance [53]. In the study by Paris et al. an elevated REE (+79 kcal/d) was observed in an intraindividual comparison of six obese adults between low EF and high EF (70-100 min exercise at 50% VO_2max) during energy balance [52]. These studies included an even smaller sample size as the current study and were not conducted in a metabolic chamber. Thus, with exception of the study of Goran et al., in which TEE was measured with doubly labeled water the equal energy balance cannot be fully confirmed. This current study, therefore, provides an important

contribution to investigating the impact of EF on SEE. The mode of physical activity between the current study and the studies mentioned above differs in respect to exercise intensity and duration. The current study adds, that SEE is also increased at higher EF when physical activity with low intensity is performed.

An increased SNS activity was previously considered as a mechanism for increased REE and SEE [156]. It has been shown, that a greater REE in regular exercising older adults is linked to β -adrenergic receptor stimulation [157]. Bell et al. found that this greater β -adrenergic receptor stimulation is caused by a chronically elevated EF and may be mediated by a higher SNS activity, as measured by skeletal muscle sympathetic nerve activity [155]. Norepinephrine, as an indirect measurement of SNS activity, was elevated at high EF compared to low EF after excluding one single outlier and was positively correlated to REE in the study by Bullough et al. [154]. This correlation is consistent with the results of the current study (Figure 5). However, no difference in the mean values of norepinephrine was found between the EF levels. Therefore, it remains unclear if the differences in SEE in the current study can be attributed to differences in SNS activity.

It is known, that physical activity can increase energy expenditure during the post-exercise period above pre-exercise levels. This effect is referred to as excess post-exercise oxygen consumption (EPOC) and involves a rapid as well as a prolonged component [158]. A significant prolonged effect of EPOC appears to exist above a threshold of 40-50% VO_2max [159], can last over 24 h after the exercise bout [160] and has shown to be approximately 5-10 % of REE [161]. Elevated REE values with a high EF in the studies mentioned above [52,53,154] might be in part the result of prolonged EPOC because REE was measured in time frames between 12 and 21h after the last exercise bout and exercise intensity was between 50%-75% VO_2max . Contrary to the other studies [52,53,154], which investigated medium- to high-intensity exercise sessions (50%-75% VO_2max) the current study included low-intensity physical activity (about < 40% VO_2max) and the duration of a single session was longer with up to 110 min. EPOC is mainly determined by exercise intensity rather than duration, and EPOC resulting from exercise durations less than 3 h with less than 55% VO_2max is limited to the training period itself [162]. Therefore, the increase in SEE in the current study was rather independent of a prolonged effect of EPOC.

DIT is also influenced by the level of EF because energy intake is increased with higher EF level, and DIT (in kcal) increases linearly with energy intake [118,125]. Not only the magnitude of DIT, but also the duration of DIT increases linearly with higher energy intake [117,125]. It has been shown, that after consumption of a single mixed liquid meal (about 981-1,127 kcal) it lasts over 8 h until metabolic rate prior to the meal was reached again [125]. In the current study, the average energy content of a single meal was 948 kcal at medium EF and 1093 kcal at high EF during EB. Therefore, it can be presumed, that the increase in SEE with a higher EF (during energy balance and overfeeding) is a confounding effect of prolonged DIT. Thus, the SEE values at a higher EF level during energy balance and overfeeding are maybe overestimated. Contrary to this assumption, SEE at low EF during OF was apparently not increased compared to medium EF during EB (Figure 3), although energy intake was with 2974 kcal/d and 2845 kcal/d almost similar between these two intervention days. Paris et al. showed, that a high EF compared to a low EF led to a higher DIT as a percentage of energy intake in five out of six participants following a standardized breakfast [9]. This suggests, that energy dissipation in the DIT component of TEE might exist in response to a higher EF and could help to improve the regulation of energy balance. It was found, that the extent of DIT (% of energy intake) is linked to SNS-activity [163] and there is further evidence, that DIT can be enhanced when exercise is performed after the meal compared to exercise before a meal or compared to no exercise [164]. In the present study, however, no increased SNS-activity (measured as catecholamine excretion) was observed at a higher EF (*see Results*). The calculation of DIT (% of energy intake) at medium and high EF in the current study could have helped to clarify the question but the calculation was not possible because of methodological drawbacks (*see Chapter II, discussion of methods*). Further studies on EF should investigate if a higher EF leads to energy dissipation within the DIT component.

In contrast to EB and OF, there was no increase in SEE during CR at medium or high EF. Thus, it appears that the presence of a negative energy balance prevents the high EF-induced SEE increase. This is in accordance with the results by Bullough et al., who found no increase in REE or elevated norepinephrine concentrations with high EF during caloric restriction [154]. Since thyroid hormones are sensitive to energy imbalances [165] and thyroid hormones can have a major influence on the regulation of energy metabolism and

interact with the SNS [166], low thyroid hormone levels during CR might have hampered the increase in SEE with higher EF. In contrast, in a randomized controlled trial the reduction of fat mass was accompanied by decreased triiodothyronine only when it was achieved by caloric restriction, but not when fat loss was induced by exercise [167]. In the study by Goran et al. (1994) a higher REE was observed at high EF during negative energy balance (however, only two participants were investigated) compared to a low EF during energy balance [53]. Maybe no differences in SEE occurred during CR because the duration of DIT was shorter than during EB and OF at medium and high EF and was confined to the daylight phase of the intervention day. Different from EB and OF, a confounding effect of DIT on SEE and consequently overestimation of SEE is not probable during CR, because energy intake was smaller.

In order to address the hypothesis if a higher EF can promote adaptive thermogenesis in response to overfeeding or prevent adaptive thermogenesis in response to caloric restriction, energy expenditure parameters were compared between OF and EB as well as CR and EB.

The current study does not support the hypothesis of adaptive thermogenesis in response to overfeeding because no difference in, TEE, SEE and EE_{active} , EE_{inactive} at all EF levels was observed during OF compared to EB. Therefore, there was no synergistic effect of overfeeding and higher EF on the occurrence of energy dissipation within one day and 25% overfeeding.

During CR, TEE and SEE, EE_{active} and EE_{inactive} were only decreased with medium EF compared to EB, but not with low EF. Because of the inconsistency of these results, the current study was not able to assess adaptive thermogenesis in response to caloric restriction, as it was not possible to compare the extent of the decrease in TEE between the EF levels. This may be attributed to the low number of subjects, that resulted in a low post hoc calculated statistical power of 13 % for the difference of TEE at low EF between EB and CR (paired t-test, α -level of 0.05). In addition, in the current study, only one day of a 25% caloric restriction was investigated. Müller et al. addressed the kinetics of adaptive thermogenesis and found that a decrease became significant after 3 days of a 50% caloric restriction diet [168]. This implies, that the caloric restriction was too slight and the

duration too short. An overall interaction, between energy balance condition and EF level, however, was observed for TEE.

When calculating the energy intake for the CR and OF intervention days, it was not taken into account, that with a lower (during CR) or higher (during OF) energy intake, the absolute values of DIT (kcal/d) decrease during caloric restriction and increase during OF. In the case of CR, relative energy balance was higher than calculated (> 75%) at all EF levels (Table 2). This can be explained by the smaller contribution of DIT to total energy expenditure during caloric restriction (because of a about 25% lower energy intake as during the measurement of energy expenditure in the pre-study test, that was used to calculate energy intake). Therefore, the higher than calculated rEB cannot be considered as evidence for the existence of adaptive thermogenesis. Likewise, in the case of OF, lower than calculated relative energy balance values (< 125%) could have been expected at all EF levels because of a higher DIT (kcal/d) with OF compared to the pre-study test. This was, however, not observed and the values for relative energy balance during overfeeding (Table 2) suggest that DIT did not show a direct proportional increase with increased food intake during overfeeding, independent of EF.

Impact of energy flux on Macronutrient oxidation

In accordance with the proposed hypothesis relative fat balance was improved at medium EF (EB: +17%, OF: +14%) and high EF (EB: +23 %, OF: +17%) compared to low EF during EB and OF (Table 3). This was achieved by a higher 24-h fat oxidation with increased EF.

In general, regulation of fat metabolism involves FFA transport across the muscle cell membrane, transport of FFA in the cytoplasm, intramuscular triacylglycerol synthesis and degradation, transport of FFA across the mitochondrial membrane, the regulatory system within the β -oxidation pathway and mitochondrial density as well as capacity to oxidize fat [169]. Exercise can promote fat oxidation by affecting these regulatory sites. For example, exercise induces catabolic pathways by activation of AMP-activated protein kinase (AMPK), known as a master energy sensor. AMPK activation promotes the entry of fatty acids into the mitochondria through the decrease of malonyl-CoA and subsequent

attenuation of the inhibition of the enzyme carnitine palmitoyltransferase 1 (CPT-1) [170]. Fatty acid translocase (FAT)/CD36, a fatty acid binding protein involved in fatty acid uptake, was found to be increased even after a single exercise bout [171]. FAT/CD36 was found to be located not only in the plasma membrane but also in the mitochondrial membrane and mitochondrial content of FAT/CD36 was increased after 30 min of electrical stimulation of muscle compared with a non-stimulated control [172]. Therefore, FAT/CD36 is suggested to play an important role in the short-term regulation of exercise-induced fat oxidation.

It is known, that in particular physical activity at low intensity up to 55-65% $\text{VO}_{2\text{max}}$ is able to enhance whole body fat oxidation with maximal fat oxidation rates between 47% and 52% $\text{VO}_{2\text{max}}$ [61]. A higher fat oxidation was found with low-intensity (33% $\text{VO}_{2\text{max}}$) and long duration (90 min) activity compared to moderate-intensity (66 % $\text{VO}_{2\text{max}}$) and shorter duration (45 min) exercise of similar energy expenditure [62]. These observations were, however, made during physical activity performed in the fasted state.

In contrast to the findings of the current study, several metabolic chamber studies of Melanson et al. showed no increase in 24-h fat oxidation with increased physical activity, when participants were in energy balance [59,60,173,174]. Because physical activity was performed in the postprandial state, the absence of an increase in 24-h fat oxidation in these studies was explained by an insulin-mediated decrease in lipolysis, that diminishes the supply of FFA [175]. FFA availability in plasma is a major determinant of fat oxidation in muscle during and after physical activity [176]. In addition, it has been suggested that increased insulin concentrations can directly inhibit the transfer of fat through the muscle cells and mitochondrial membranes and hence inhibit intramuscular triacylglycerol oxidation [169,177]. This is confirmed by a recent meta-analysis, that has shown a higher fat oxidation during aerobic exercise (≤ 120 min) performed in the fasted state when compared with aerobic exercise after the ingestion of a meal [178]. Another study found that only when exercise was performed before breakfast (fasted state), 24-h fat oxidation was increased [58]. Schrauwen et al. (1997) investigated 12 healthy subjects in a metabolic chamber and showed that when participants underwent a glycogen lowering exercise session the day prior the stay in the metabolic chamber, fat oxidation rapidly increased in response to a high-fat diet during energy balance [179]. In line activation of AMPK is inversely correlated to glycogen content [170].

These findings emphasize the importance of the timing of physical activity in relation to food intake and indicate that fat oxidation can only be enhanced when physical activity is performed in a fasted state when glycogen stores in the muscle are depleted.

However, in the current study FFA_{tAUC} was increased at medium EF during EB and at medium and high EF during OF compared to low EF (Table 3). This suggests, that lipolysis was not diminished (despite a presumably higher insulin level with higher food intake) and supports the observed increase in 24-h fat oxidation at higher EF. Notably, all three physical activity sessions were performed during the postprandial phase (within 40 min after the beginning of the meal). FFA availability seems not to be a limiting factor for fat oxidation in the current study, as fat oxidation was not further increased at a higher EF during CR compared to EB, although FFA_{tAUC} was increased. In summary, the increased 24-h fat oxidation and relative fat balance at higher EF occurred despite presumable higher insulin levels with higher energy intake and is a unique finding that has not been systematically investigated before.

The studies of Melanson et al. compared a day with a single exercise bout of 40-60 min or of 400 kcal energy expenditure and 40-70% $\text{VO}_{2\text{max}}$ to a sedentary control day. The exercise was performed by cycling on a stationary ergometer and participants were lean sedentary, endurance-trained as well as obese sedentary men and woman [59,60,173,174]. The main difference between the current study and these studies is the duration (3 x 55 min/d and 3 x 110 min/d), the mode of physical activity (walking vs. cycling) and the frequency of physical activity throughout the day (one vs. 3 sessions). Maybe the discrepancies in the results can be attributed to these differences. Physical activity was performed at three different time points during the day and when summed for a much longer duration as in the previous studies (165 min - 330 min vs. 60 min). This suggests that not only meal timing relative to the activity period is an important factor to consider, but also the frequency and duration of physical activity may affect 24-h fat oxidation and subsequently fat balance. It can be hypothesized, that the duration of physical activity in the other studies was too short to affect 24-h fat oxidation. This assumption is supported by Smith et al., who investigated the adaptation to a high fat diet and observed an accelerated increase in 24-h fat oxidation to a high fat diet after 1 day, when physical activity was performed by walking 2-3 times a day on a treadmill with 4.8 km/h to reach a PAL of 1.8 [180]. The frequency, mode, duration, and intensity of physical

activity in this study is very similar with the physical activity intervention in the current study. It was previously suggested, that the rate of fat oxidation increase with proceeded exercise duration and is maybe caused by a reduction in muscle glycogen with prolonged physical activity [181,182]. In Addition, walking instead of cycling has shown to have a greater impact on fat oxidation (higher fat oxidation rates), maybe because walking involves the recruitment of a larger muscle mass than cycling [183].

However, it must be considered that the differences in fat oxidation in the current study were found compared to a very sedentary state (low EF), that may occur rarely in real life. Step count for the low EF days was on average between 415-482 steps/day (Table 2) and fat oxidation was 51 ± 14 g/d during EB and 41 ± 11 g/d during OF (Table 3). These values for fat oxidation were lower compared to the control day in the study of Melanson et al. [174], in which no difference in 24-h fat oxidation was found comparing a day with a single exercise bout to a sedentary control day. Mean 24-h fat oxidation on the control day of this study was with 86.5 g/d already relatively high [174]. Relative fat balance in the current study was only increased from low to high or from low to medium EF, but not from medium to high EF. Nevertheless, fat oxidation further increased from medium to high EF and reached 102 ± 27 g/d at high EF during energy balance.

Remarkably, 24-h fat oxidation during EB increased at medium and at high EF to the same level as during CR (Table 3). Thus, fat oxidation as a percentage of TEE reached the same magnitude with medium and high EF during EB when compared with CR. Relative fat balance was improved at a higher EF during EB and during OF with an increase from 54% to 77% for EB and 35 % to 52% for OF. These results occurred despite the fact, that fat oxidation is not directly linked to fat intake as it is the case with carbohydrate oxidation [184]. However, fat intake was still higher than fat oxidation at high EF. Therefore, the impact of increased EF on fat metabolism, to maintain or even reduce body fat mass, remains unclear.

In contrast to EB and OF, during CR 24-h fat oxidation was equal to fat intake independent of EF level and relative fat balance was not further improved at higher EF (Table 3). Thus, there was no synergistic effect between caloric restriction and physical activity on relative fat balance. Although 24-h fat oxidation was higher at high EF compared to low EF. Two randomized controlled intervention studies showed greater

body fat loss after 6 months [185] and one year [186] when caloric restriction diets were combined with physical activity compared to a caloric restriction diet alone. Based on the current study this can rather be attributed to a greater negative energy balance with the combined approach than to an impact of physical activity independent of energy balance.

Relative carbohydrate balance and carbohydrate oxidation as a percentage of TEE did not differ between the EF levels at all energy balance condition (Table 3). Therefore, carbohydrate oxidation was still the main source of energy supply without a shift in contribution at medium and high EF.

24-h protein oxidation was not as tightly coupled to protein intake as expected from the literature [184]. A higher 24-h protein oxidation than intake was observed at low EF during EB. Relative protein balance shifted from a negative ($> 100\%$) to a positive ($<100\%$) balance as EF became higher. This is in accordance with the study of Melanson et al. (2009), in which a higher protein balance has been found when the participants were exercising compared to a sedentary control day [60]. The increase in fat oxidation with higher EF was therefore accounted for by a decrease in protein oxidation. A higher EF might promote protein anabolic processes, that helps to maintain or even increase muscle mass. Vice versa protein oxidation was not adequately diminished in response to a decrease in protein intake with very sedentary behavior and could, therefore, enhance muscle mass loss. During CR relative protein balance was negative with all EF levels but was less negative with higher EF. These results suggest that muscle mass losses during short-term caloric restriction can be decreased with a high EF. This is in line with a 12-week and a 4-month intervention study showing that aerobic exercise has attenuated the loss of skeletal muscle during caloric restriction [187,188].

Strength and limitations of the study

The strength of this study is that a very strictly controlled protocol was used, where the participants were supervised during the whole study period, except for the time during washout days, when the participants were allowed to go home. An important advantage of this study is, that in addition to the intraindividual comparison of different

levels of EF during energy balance, participants were also examined during caloric restriction and overfeeding.

However, there are several limitations to the present study that need to be addressed. First, energy balance was slightly but significantly positive at low EF during the EB condition. The deviation from energy balance ranged for the individual participants between -31 kcal/d and +277 kcal/d, resulting in an average deviation of +75 kcal/d. Although this was statistically significant, the literature suggests that energy balance can be assumed, when the deviation is not higher than 4% [189]. Only two participants out of 11 had a higher deviation with a +10 % and +11 % positive energy balance at low EF. However, these two participants were no obvious outliers with respect to fat oxidation parameters, thus it was assumed that the overall outcome was not influenced.

Another limitation is, that no decrease in energy expenditure at low EF during CR was observed. Therefore, the study failed to investigate, whether a higher EF can prevent adaptive thermogenesis due to caloric restriction. A higher energy deficit (resulting in larger differences in TEE between the conditions) or more participants would have helped to clarify the results.

Energy balance during CR and OF were calculated to be 25 % lower or higher for each EF level than during EB. This resulted in a higher absolute energy deficit and surplus with higher EF. For example, in the case of caloric restriction, the energy deficit was greater in terms of absolute values with increased EF because caloric restriction was calculated to be the same proportion of energy expenditure at all EF levels. Thus, caloric restriction in absolute values at low EF was less (-475 \pm 184 kcal/d) compared to the caloric restriction with medium EF (-533 \pm 130 kcal/d) and high EF (-663 \pm 132 kcal/d). Greater energy imbalances in terms of absolute values may have distorted the comparison of outcome parameters during CR and OF.

Conclusion

This study has shown, that a higher energy flux might have beneficial effects on body weight regulation during energy balance and overfeeding because it increased sleeping energy expenditure and improved relative fat balance. In particular, this study shows that a higher EF can attenuate the adverse effects of short-term overfeeding on fat metabolism. In contrast during CR, 24-h fat oxidation was equal to fat intake independent of energy flux. Relative fat balance was not further improved and SEE did not increase at higher EF during CR. Therefore, no short-term synergistic effect of caloric restriction and physical activity on body weight regulation can be assumed. Although, 24-h fat oxidation was higher with high EF compared to low EF.

Chapter V

Concluding discussion

This thesis investigated the impact of meal skipping and energy flux on energy and macronutrient metabolism under controlled energy balance conditions by using a metabolic chamber. The first part of the thesis showed that when using metabolic chambers to study energy- and macronutrient metabolism, thorough considerations must be made in terms of the metabolic chamber environment (room ventilation and position of analyzer unit), the additional devices used (e.g. air conditioner) as well as the study protocol in order to obtain data of good quality.

Usually, metabolic chamber studies are used to investigate the intervention effect independent of changes in energy balance [31,97]. This is very important for understanding the underlying mechanisms of interventions on body weight regulation. However, the state of energy balance has to be considered as an artificial situation [173], since in real life there is rather a continuous change between short-term caloric restriction and overfeeding [63,190]. This transient energy deficits or surpluses can be observed over a time period of single days or even within a day on an hourly basis, e.g. short-term positive energy balance occurs after a meal and negative energy balance can

precede a meal [191]. The definition of energy balance, therefore, depends on the investigated time frame. When energy metabolism is examined under conditions of energy balance (e.g. over one day), the obtained results represent the sum of the impact of episodes of negative and positive energy balance that offset each other. The current study on energy flux (*Chapter IV*) also investigated the impact of EF during one-day caloric restriction and overfeeding, as these are the situations in which the metabolism is challenged. Controlled studies under energy imbalance could help to improve the understanding of the regulation of energy- and macronutrient metabolism as well as to identify the risk factors for the development of obesity, diabetes or cardiovascular disease.

In a metabolic chamber study, it is important to appropriately adjust energy intake to the targeted condition of energy balance. There are several studies, that failed to accurately calculate energy intake and consequently did not achieve energy balance by using predictive equations [184] or by measuring or estimating REE and multiplying it with an estimated mean PAL for all participants [130,192,193]. The challenge to predict energy requirement is owed to the fact that, physical activity is highly variable not only in free-living conditions [194] but also in the confined setting of a metabolic chamber [31,97]. In Addition, the accuracy of published predictive equations for REE are rather poor [195]. Schrauwen et al. demonstrated that measuring EE for 24-h in a metabolic chamber prior to the actual intervention and using this EE to calculate energy requirement, reduced the deviation from energy balance compared to using SEE and an estimated PAL [97]. The inclusion of an activity protocol further improved the goal of energy balance. Schrauwen et al. pointed out that a deviation of 4% can be considered as sufficient to indicate a participant in energy balance because calculating energy intake by using food composition tables has an accuracy of about 2% and using metabolic chambers as a method to measure energy expenditure also has an accuracy of about 2% [189].

In this thesis for the study on meal skipping (*Chapter III*) energy requirement was assessed by measured REE multiplied with a PAL of 1.35. In the study on energy flux (*Chapter IV*) energy requirement was calculated by measuring TEE in the metabolic chamber for the three different EF levels prior to the intervention days. When comparing the magnitude of deviation from real energy balance between the two studies (control day of the meal skipping study compared to low EF during EB of the energy flux study) the

results of Schrauwen et al. can be confirmed. In the study on meal skipping a maximal deviation of 22% from energy balance was found, but the maximal deviation in the EF study was only about 11%. This approach allows a more accurate adjustment of the energy balance condition, although it is labor-intensive and time-consuming. De Jonge et al. suggested to measure energy expenditure during the first 3 h or 7 h of the stay in the metabolic chamber and to use this value as a basis to adjust energy intake or physical activity to achieve energy balance [196]. Lam et al. developed predictive equations for sedentary TEE in a metabolic chamber and compared them to the approach of de Jonge et al [31]. The developed equations showed to be better than the usually used equations and energy prescription was further improved by adjustment of food intake using the 3h or 7h energy expenditure measurement in the metabolic chamber. This refinement was attributed to a better estimation of activity energy expenditure [31].

In summary, in order to achieve well-controlled conditions for energy balance, the use of measured energy expenditure in a metabolic chamber is important and interventions during energy imbalance can contribute to research on body weight regulation and metabolic health.

The study on meal skipping (*Chapter III*) has shown that breakfast skipping increases 24-h fat oxidation during energy balance, however, this was accompanied by a decrease in insulin sensitivity at lunch. Metabolic flexibility was therefore impaired by breakfast skipping independent of energy balance. In addition, FFA_{TAUC} was elevated on the breakfast skipping day compared to the three-meal control day. The concept of metabolic flexibility describes a healthy metabolism as a system, that has a high adaptability to changes in nutritional condition, e.g. shift of fuel selection from fat oxidation to glucose oxidation in response to insulin stimulation [197]. Metabolic inflexibility conversely, is characterized by a mixed oxidation of the three macronutrients and blunted fuel switch in response to changes in nutritional and physiological conditions [147]. Muoio et al. found, that situations of unabated substrate competition that occur with chronic overfeeding lead to mitochondrial indecision and thus metabolic inflexibility [147]. Besides the fact that FFAs are an important source for fat oxidation, the occurrence of free fatty acids

impairs insulin sensitivity because FFAs compete with glucose for mitochondrial uptake and oxidation [198,199].

In the study on meal skipping, the energy content of the skipped meal was compensated for in the lunch and dinner meal. Thus, the energy content of these meals (50% of energy requirement each) was very high. The phase after lunch, therefore, can be considered as a period of short-term overfeeding, where the mitochondrial system was challenged by the simultaneous availability of dietary glucose and FFA from the preceding fasting period. According to Muoio et al., human physiology has developed under conditions of dramatic fluctuations in energy intake and the human metabolism had to cope with periods of fasting, that were followed by refueling [147]. In this context, the concept of intermittent fasting (time restricted eating, alternate day fasting), has become of interest and might have beneficial effects on long-term health [200]. This concept seems to be in contrast to the findings of the meal skipping study in this thesis. However, it is important to consider that the human physiology has also developed under the condition of a high EF [13]. In the current study, breakfast skipping was investigated during a sedentary condition. Goodpaster and Sparks suggested in a recent review that insulin resistance might be rather an adaptive response than pathobiology under certain conditions as e.g. elevated fatty acids oxidation with prolonged fasting [197] because insulin resistance in response to lipid oversupply was observed, despite normal function of insulin signaling and the absence of intramyocellular fat accumulation [201]. It was concluded that the pathogenesis of insulin resistance is context specific and in case of prolonged fasting is more likely caused by substrate competition than by impaired insulin signaling [202]. Mitochondrial performance and fat oxidation are enhanced in endurance-trained subjects and are related to better preservation of non-oxidative glucose disposal in response to lipid overload [201]. Therefore, a higher physical activity might attenuate substrate competition induced insulin resistance. In Addition, the level of physical activity is strongly associated with metabolic flexibility and metabolic flexibility can be modulated by physical activity [203]. Insulin action is enhanced by single exercise bouts [204] and physical activity stimulates insulin-independent glucose uptake by translocation of GLUT 4 transporter to the muscle cell surface via activation of AMPK [205]. In particular, exercise of low-intensity has shown to increase both fat oxidation and insulin sensitivity [206]. This was suggested to be explained by improvements in the mitochondrial capacity to oxidize

fat by relying on intramyocellular lipids and thus by the reduction of intramuscular lipid metabolites like diacylglycerol or ceramides that may interfere with insulin signaling. In addition, improved mitochondrial fatty acid uptake was observed in response to endurance exercise training [207,208]. The study on energy flux (*Chapter IV*) has shown to improve fat oxidation at a higher EF even during overfeeding. Maybe, a high EF state, therefore, has the potential to attenuate or even remove the metabolic disadvantages of breakfast skipping.

Altogether, the two studies of this thesis revealed that challenging situations for mitochondrial metabolism such as episodes of short-term overfeeding that follow prolonged fasting should be coupled with simultaneously increased physical activity in order to attenuate adverse metabolic outcomes. Especially physical activity after lunch and dinner, when breakfast was skipped could attenuate the metabolic risk.

This thesis emphasizes the importance of physical activity in daily life and suggests that the metabolic outcome of meal skipping, in particular, breakfast skipping, depends on the level of energy flux and the timing of physical activity.

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Contributions to clinical trials

Alessa Nas performed the research studies and analyzed the research study data in Chapter III and IV.

Prof. Dr. Dr. Anja Bosy-Westphal (Kiel University) designed, supervised and co-performed the research studies in Chapter III and IV.

Franziska A. Hägele (Kiel University) co-analyzed the glucose metabolism data in Chapter III and co-performed the research study in Chapter III and IV.

Dr. Julia Kahlhöfer and Dr. Judith Keller (University of Hohenheim) co-performed the research study in Chapter III.

Franziska Büsing (Kiel University) co-performed the research study in Chapter IV.

Dr. Mario Hasler (Kiel University) provided statistical support for data analyzes in Chapter IV.

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Originalarbeiten

Hägele FA, Büsing F, **Nas A**, Aschoff J, Gnädinger L, Schweiggert R, Carle R, Bosy-Westphal A. High orange juice consumption with or in-between three meals a day differently affects energy balance in healthy subjects. *Nutrition & Diabetes* 2018 Apr25;8(1):19

Büsing F, Hägele FA, **Nas A**, Döbert LV, Fricker A, Dörner E, Podlesny D, Aschoff J, Pöhl T, Schweiggert R, Fricke WF, Carle R, Bosy-Westphal A. High intake of orange juice and cola differently affects metabolic risk in healthy subjects. *Clinical Nutrition* 2018 Mar 3. pii: S0261-5614(18)30093-1 [*in press*].

Nas A, Mirza N, Hägele F, Kahlhöfer J, Keller J, Rising R, Kufer TA, Bosy-Westphal A. Impact of breakfast skipping compared with dinner skipping on regulation of energy balance and metabolic risk. *The American Journal of Clinical Nutrition* 2017 Jun;105(6):1351-1361

Kongress- und Tagungsbeiträge

Nas A, Hägele FA, Büsing F, Bosy-Westphal A. Impact of daylong changes in energy flux on sleeping energy expenditure and fat oxidation. Postervortrag, *11th International Symposium on In Vivo Body Composition Studies 2018*, New York, USA

Nas A, Hägele FA, Büsing F, Bosy-Westphal A. Einfluss des Energy-Flux auf die Regulation des Körpergewichts während ausgeglichener Energiebilanz, Über- und Unterernährung. Kongressvortrag, *Ernährung 2018*, Kassel, Deutschland

Nas A, Hägele FA, Büsing F, Bosy-Westphal A. Impact of energy flux on sleeping energy expenditure and fat oxidation during energy balance, caloric restriction and overfeeding. Postervortrag, *European Congress on Obesity 2018*, Wien, Österreich

Nas A, Hägele FA, Büsing F, Bosy-Westphal A. Impact of energy flux on energy expenditure and macronutrient partitioning during energy balance, caloric restriction and overfeeding. Kongressvortrag, *Kongress der Deutsche Gesellschaft für Ernährung 2018*, Stuttgart, Deutschland

Nas A, Hägele FA, Büsing F, Schutz Y, Bosy-Westphal A. Impact of energy flux on sleeping metabolic rate and fat balance during energy balance, caloric restriction and overfeeding. Postervortrag, *International Conference on Recent Advances and Controversies in Measuring Energy Metabolism 2017*, Fribourg, Schweiz

Nas A, Hägele FA, Kahlhöfer J, Keller J, Rising R, Bosy-Westphal A. Impact of breakfast skipping vs. dinner skipping on regulation of energy balance and metabolic risk. Postervortrag, *European Congress on Obesity 2017*, Porto, Portugal

Nas A, Kahlhöfer J, Hägele FA, Karschin J, Bosy-Westphal A. Einfluss von Mealskipping auf die Makronährstoffoxidation und den 24h-Energieverbrauch bei gesunden Probanden. Kongressvortrag, *Kongress der Deutsche Gesellschaft für Ernährung 2017*, Kiel, Deutschland

Nas A, Kahlhöfer J, Hägele FA, Karschin J, Bosy-Westphal A. Impact of the timing of meal skipping on 24h-macronutrient oxidation in healthy adults. Postervortrag, *European Obesity Summit 2016*, Göteborg, Schweden

Nas A, Kahlhöfer J, Hägele FA, Karschin J, Bosy-Westphal A. Einfluss von Mealskipping bei ausgeglichener Energiebilanz auf die 24h-Makronährstoffoxidation bei gesunden Probanden. Kongressvortrag, *Ernährung 2016*, Dresden, Deutschland

Stipendien und Auszeichnungen

2018	Reisestipendium der GSK Stiftung, Vortragsreise <i>11th International Symposium on In Vivo Body Composition Studies 2018</i> in New York, USA
2018	Posterpreis , Kongress <i>Ernährung 2018</i> in Kassel, Deutschland
2018	Reisestipendium des Instituts Danone „Ernährung für Gesundheit e.V.“, Vortragsreise <i>European Congress on Obesity 2018</i> in Wien, Österreich

Ort und Datum

Unterschrift

**Eidesstattliche Versicherung gemäß § 7 Absatz 7 der Promotionsordnung
der
Universität Hohenheim zum Dr. rer. nat.**

1. Bei der eingereichten Dissertation zum Thema: „Metabolic chamber studies on energy- and macronutrient metabolism: Impact of meal skipping and energy flux“ handelt es sich um meine eigenständig erbrachte Leistung.
2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.
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